Role of the Lipoxin A4 Receptor in Inflammation

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To my father Prof. Manfred Waechter

CONTENTS

SUMMARY

The lipoxin A4 receptor (ALXR) is expressed in almost all leukocytes and plays an important role in host defense and inflammation such as rheumatoid arthritis and asthma. The receptor binds structurally diverse agonistic ligands, including peptides and lipid mediators, which mainly regulate chemotaxis and activation of polymorphonuclear neutrophils (PMN) and monocytes that enter the inflamed tissue. The regulation of ALXR is poorly described but a few regulators, involved in inflammation, have been identified to upregulate the expression of ALXR. For instance ALXR is regulated in enterocytes by cytokines such as interleukin-6 (IL-6) interleukin-13 (IL-13) and interleukin-4 (IL-4). However, little is known about its own transcriptional and translational regulation in myeloid cells such as monocytes and macrophages. In this thesis I aim at better understanding the transcriptional regulation of the ALXR in macrophages to delineate its role during inflammation.

I identified two promoter regions separated by 224 bps which drive the expression of the ALXR in macrophages. Both promoter regions increased transcription in a reporter assay and the basal transcription factors, OCT1 and SP1, were shown to bind the first and the second promoter, respectively, and to transactivate transcription. To investigate the ALXR mRNA expression in monocytes and macrophages, I measured basal expression of the ALXR mRNA during differentiation of monocytes to macrophages and stimulated mRNA expression in macrophages. While monocytes expressed high levels of ALXR mRNA, differentiation into macrophages abrogated ALXR expression. Stimulation of macrophages with a set of cytokines revealed that only IFNγ increased ALXR expression to levels similar to the ones detected in monocytes. This upregulation by IFNγ is in part mediated by IRF1 interacting with an IRSE transcription factor binding site located in the first promoter region of ALXR.

These data support that the ALXR plays a role in the chemotaxis of monocytes, which results from a gradient of pro-inflammatory or anti-inflammatory ligands. Depending on the ligand and the environment of the invaded tissue, the monocyte may then differentiate into either an M1 or a M2 macrophage. Since IFNγ is known to induce the differentiation of macrophages into pro-inflammatory M1 type macrophages, my data suggest that in the early phase of inflammation only M1 macrophages could further be triggered by the pro-inflammatory ligands of ALXR to generate inflammatory regulators and to recruit PMN and monocytes that recognize and kill pathogen. On the other hand, when anti-inflammatory lipid ligands occur at the site of inflammation, these M1 macrophages may re-differentiate into M2 macrophages, and play a role in restoring tissue homeostasis.

ZUSAMMENFASSUNG

Der Lipoxin A4 Rezeptor (ALXR) wird von nahezu allen Leukozyten exprimiert und erfüllt eine wichtige Funktion in der Wirtsabwehr und in entzündlichen Erkrankungen wie rheumatoide Arthritis und Asthma. Der Rezeptor bindet strukturell unterschiedliche agonistische Liganden. Dazu gehören Peptide und Lipidmediatoren, welche vor allem die Chemotaxis und die Aktivierung von polymorphkernigen neutrophilen Granulozyten (PMN) und Monozyten regulieren, die in entzündetes Gewebe einwandern. Die Regulierung von ALXR ist kaum beschrieben. Nur, einzelne an Entzündungen beteiligte Regulatoren die die Expression von ALXR erhöhen, wurden identifiziert. In Enterozyten wird ALXR beispielsweise von Zytokinen wie Interleukin-6 (IL-6), Interleukin-13 (IL-13) und Interleukin-4 (IL-4) reguliert. Jedoch ist wenig über seine transkriptionnelle und translationelle Regulierung in myeloiden Zellen wie Monozyten und Makrophagen bekannt. Das Ziel meiner Arbeit ist, ein besseres Verständnis der transkriptionalen Regulierung des ALXRs in Makrophagen zu erhalten, um dessen Funktion bei einer Entzündung zu verstehen.

Ich habe zwei Promoterregionen, getrennt durch 224 Basenpaare, identifiziert, welche die Expression von ALXR in Makrophagen vermitteln. Beide Promoterregionen erhöhten die Transkription in einem Reporter-Assay, und es konnte gezeigt werden, dass die basalen Transkriptionsfaktoren OCT1 und SP1 an den ersten beziehungsweise den zweiten Promoter binden und die Transkription aktivieren. Um die Expression des ALXR in Monozyten und Makrophagen zu untersuchen, habe ich die basale Expression der ALXR-mRNA während der Differenzierung der Monozyten zu Makrophagen und die mRNA-Expression in stimulierten Makrophagen gemessen. Während Monozyten ALXR-mRNA in hoher Konzentration exprimierten, führte die Differenzierung zu Makrophagen zur Einstellung der ALXR-Expression. Stimulierung von Makrophagen mit verschiedenen Zytokinen zeigte, dass lediglich IFNγ die ALXR-Expression auf Konzentrationen ähnlich der in Monozyten gemessenen erhöhte. Diese Hochregulierung durch IFNγ wird zum Teil durch die Interaktion von IRF1 mit einer Bindungsstelle für den Transkriptionsfaktor IRSE vermittelt, welche sich in der ersten Promoterregion für ALXR befindet.

Diese Daten bestätigen einen Einfluss von ALXR auf die Chemotaxis von Monozyten, die auf einen Gradienten von pro- oder anti-inflammatorischer Liganden zurückzuführen ist. Die Monozyte könnte abhängig vom Liganden und vom Milieu des invadierten Gewebes entweder in eine M1- oder eine M2-Makrophage differenzieren. Da IFNγ dafür bekannt ist, die Differenzierung von Makrophagen zu pro-inflammatorischen Makrophagen vom Typ M1 zu induzieren, legen meine Ergebnisse nahe, dass in der frühen Phase der Entzündung nur M1-Makrophagen durch die pro-

inflammatorischen Liganden von ALXR aktiviert werden können. Diese aktivierten M1-Makrophagen könnten inflammatorische Regulatoren bilden und PMN und Monozyten rekrutieren, welche Pathogene erkennen und vernichten. Wirken jedoch anti-inflammatorische Lipidliganden an der Entzündungsstelle, könnten diese M1-Makrophagen zu M2-Makrophagen redifferenzieren und mithelfen, die Homöostase des Gewebes wiederherzustellen.

1 INTRODUCTION

1.1 **Inflammation and innate immune response**

The definition of inflammation is the overall phenomenon of the organism defence against traumatism, irritation and infection characterized by dolor, calor (heat), tumor (swelling) and rubor (redness). It is an attempt to protect the organism in removing the danger and in restoring tissue physiological function through the healing process. Inflammation is generally considered beneficial and has evolved as an adaptive response for restoring homeostasis [1]. In the absence of inflammation, tissue would be gradually damaged, which could lead to death of the organism. Acute inflammation lasts from a few hours to a few days and requires the activation of the innate and later the adaptive immune response. It consists of increases in the blood flow, activation of the plasma cascade and augmentation of permeability across blood capillaries. It is characterized by a quick migration of white blood cells, named leukocytes, from the blood to the injured sites where they elicit an inflammatory response. However when dysregulated inflammation becomes a pathological state lasting months to years, it is described as chronic inflammation. Much less is known about the causes and mechanisms of chronic inflammation, which occurs in several diseases such as asthma, inflammatory bowel disease, rheumatoid arthritis and atherosclerosis.

1.1.1 Danger signals and recognition

The first line of the host defence in inflammation is the epithelial barrier that stops any pathogen to enter the tissue. If this is not avoided, innate immunity responds to the intruder via proteins in the plasma cascade and phagocytic cells like macrophages and dendritic cells which recognize special features of the pathogen, the danger-associated molecular patterns (DAMPs) [2]. DAMPs include Gram negative and Gram positive bacteria, LPS, lipopeptides, lipoteichoic acid, double stranded DNA and single stranded RNA, allergens, toxic compounds, cell death products, heat shock proteins, adenosine, urate crystals and fibrinogen [3]. DAMPs are recognized by the host cells through specific recognition receptors, the pattern recognition receptors (PRRs). They have several different functions. The phagocytic receptors, such as macrophage mannose receptors and scavenger receptors (SRs), stimulate ingestion of the pathogen they recognize. The nod-like receptors (NLRs) recognize bacteria products and the chemotatic receptors that recognize N-formylated peptides from bacteria and guide polymorphonulcear neutrophils (PMNs) to the sites of

infection. More specific receptors are the mannose binding lectin (MBLs) soluble receptors, which activate the complement system consisting of several soluble proteins interacting with each others to induce the recruitment of inflammatory cells which leads to phagocytosis and lysis of the pathogen [4]. Other PRRs are the toll like receptors (TLRs) which trigger gene expression in response to intruders and mediate their actions through signal transduction pathways such as mitogen activated protein kinase (MAPK) and transcriptional elements like nuclear factor kappa light chain enhancer of activated B cells (NF-κB), interferon regulatory factor 3 (IRF3) or activating protein (AP-1) [5]. TLRs interact also with co-stimulatory molecules such as CD14 and participate in the induction of the adaptive immune response when the innate immunity is overwhelmed.

1.1.2 Pro-inflammatory messengers and cell chemotaxis

PRRs elicit inflammatory response in cells of the immune system by producing mediators and signalling molecules such as growth factors, chemokines and cytokines. Cytokines induce expression of acute phase proteins such as C-reactive protein (CRP) from the liver that will help to eliminate the pathogen by activating the complement system. Increasing gradients of pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 12 (IL-12), interleukin 8 (Il-8) interleukin 6 (IL-6) and tumor-necrosis factor α (TNFα) attract platelets and leukocytes like PMNs, basophils, eosinophils and mast cells to the site of inflammation [6-8]. They are supported by several cysteine-cysteine type chemokines; the CCL2 (also monocyte chemotactic protein MCP-1), CCL5 (also RANTES), CXCL-12 and CX_3CL1 to recruit leukocytes [9, 10]. At the same time, endothelial cells lining the local blood vessel start to express adhesion proteins such as integrins, selectins E and P and members of the intercellular adhesion molecules (ICAM-1 and -2), vascular cell adhesion molecules (VCAM1) and platelet-endothelial cell adhesion molecule (PECAM-1) that will capture the oncoming leukocytes. The circulating leukocytes are slowed down toward the site of inflammation and begin to roll along the inner surface of the blood vessel making bonds with the selectins. The chemokines and integrins then induce firm adhesion and immobilisation of the leukocytes which release proteases to degrade the basement membranes. Leukocytes transmigrate with the help of PECAM- 1 through gaps between the endothelial cells and recognize the intruder through their cell surface receptors that can discriminate between host and pathogen cell surface particles to mediate correct immune response.

1.1.3 Inflammatory response

Recognition of the intruder by the host cells leads to the phagocytosis of the pathogen followed by its internalization into the phagosome or endocytic vacuole via cytoskeletal remodelling and organization of actin filament involving Rho, Rac and Cdc42 signaling pathway [11]. Normally these vesicles become acidified to kill the pathogen. Also they can fuse with lysosomes that contain enzymes, peptides and proteins which mediate inflammatory response. PMNs, mast cells, natural killer (NK) cells, eosinophils and basophils are particularly interesting in terms of host defence because they contain granules whose specific contents are released in the micro-environment to attack the pathogen. Granules contain myeloperoxidase (MPO) and the membrane-associated NADPH oxidases which contribute to the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are generated via a so called respiratory burst process as large volumes of oxygen are consumed at that time. Among them are superoxide (O_2^-) hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) singlet oxygen (O_2), hydroxyl radical (OH), nitryl chloride (NO_2Cl), chloramines (RNHCl), radical product (AH) and compound III ($MPO^{3+}O_2^-$). Together with MPO, they function as potent antimicrobial agents and serve as modulators of proteins, lipid kinases and phosphatases, transcription factors, including NF-κB, membrane receptors and ion channels functions [12]. However these responses can become deleterious and lead to cell deaths, tissue degradations and organ dysfunctions. Indeed NO production from the inducible nitric oxide synthase (iNOS) is harmful because it increases vascular permeability and has potent oxidative properties [13]. A derivative of NO, the peroxynitrite ($ONOO^-$), can react with lipids to initiate lipid peroxidation resulting in cell and DNA damage causing the recruitment and activation of the poly ADP-ribose polymerase. This will decrease NAD+ stores and alter the redox state of the cells. Additionally PMN-released elastases disrupt tight junctions and contribute to proteolytic damage of the tissue. Thus an extended inflammatory response is detrimental for the tissue and leads to adverse physiological consequence such as chronic inflammation.

1.1.4 Resolution of inflammation

Prolonged inflammation can cease to be beneficial and therefore has to be stopped. Thus once the pathogen is killed, resolution mechanisms start as an active process and stop the signals leading to the synthesis of the pro-inflammatory mediators such as chemokines, cytokines and cell adhesion molecules. For instance fibroblasts participate in the withdrawal of survival signals and normalize

chemokine gradients that will prevent further recruitment of leukocytes. Furthermore, other end signals, such as the vasoactive NO, released by the endothelial nitric oxidase synthase (eNOS) or the anti-inflammatory cytokines IL-10 and TGFβ reduce leukocytes adhesion and infiltration [14]. Resolution of inflammation can occur only if granulocytes such as PMNs and mononuclear cells like macrophages and lymphocytes return to normal pre-inflammation numbers and phenotypes [15]. Thus cells need to be removed from the tissue through either systemic re-circulation or local cell death. The stop signals mentioned above also support the apoptosis of leukocytes which are taken up by nonphlogistic monocytes-derived macrophages. Moreover, the anti-inflammatory glucocorticoids and annexins stimulate macrophages to take up apoptotic PMN and other leukocytes from the inflamed tissue in a pro-resolving fashion [16-18]. Once phagocytosis is complete, macrophages exit the inflamed tissue by lymphatic drainage with evidence that a small portion may die locally by apoptosis. Each of the steps leading to the return of normal tissue homeostasis are coordinated and are under the thight control of pro-resolving factors such as the lipid mediators discussed in the next chapter [19]. However, defect in these endogenous anti-inflammatory pathways may predispose the host to chronic inflammatory disease. Successful resolution will therefore limit excessive tissue damage and organ dysfunctions and give little opportunity for the development of chronic inflammation [20].

1.2 **Lipid mediators**

1.2.1 Lipid mediator in inflammation

The lipids mediators are chemical messengers that have hormone-like effects mediated by specialized receptors during the course of inflammation and are generally derived from essential fatty acids (EFA) that includes C20 arachidonic acid (AA), C20 eicosapentanoic acid (EPA) and the C22 docosahexanoic acid (DHA).

DHA and EPA are part of the omega-3 fatty acids which have in common a final carbon–carbon double bond in the *n*−3 position from the terminal methyl end of the carbon chain. In the early 80s they gained the interest of the scientific world because of their heart-health benefit. In 1984 researchers associated seafood diet with reduced triglyceride levels and blood pressure in the Eskimos population [21]. Furthermore, omega-3 fatty acids have an impact on the immune response. Long-term feeding of diets enriched in EPA and DHA decreases the production of cytokines involved in the pro-inflammatory aspect of inflammation like IL-1β, TNFα, and IL-6

[22]. A daily teaspoonful of fish oil given to healthy Danish infants for 3 months markedly increased the omega-3 fatty acid content of their red blood cell membranes. EPA is the precursor of the pro-resolution lipid mediator resolvin E_1 (RvE_1) and DHA is the precursor of resolvin D_1 (RvD_1) and protectin D_1 (PD_1). At nanomolar levels RvE_1 dramatically reduces transendothelial migration of human PMNs and thus promotes resolution of inflammation [23]. RvD1 and PD1 have pro-resolution effects especially in reduction of PMN infiltration in murine skin air pouch inflammation [24], peritonitis [25] and brain injury [26]. Additionally PD_1 promotes corneal epithelial wound healing and repair [27] and reduces airway inflammation in a mouse model of asthma [28]. These chemical mediators have thus been shown to control the magnitude and duration of inflammation in animals and function as an agonist in the different steps of the resolution of inflammation [23].

AA is part of the omega-6 fatty acids and derives from the linoleic acid which can be found in vegetable oils. Upon various physiological stimuli, AA is released by phospholipase A2 (PLA_2) from membrane phospholipids stores. Cyclooxygenases convert AA into prostaglandins, prostacyclins and thromboxanes, whereas lipoxygenases generate leukotrienes and lipoxins. During the initial phase of inflammation, the pro-inflammatory lipid mediator prostaglandins and leukotrienes are produced. Both act as a paracrine and autocrine lipid mediator. The prostaglandins are formed by most cells in our body, induce pain, vasodilatation and fever and are the first target of the non-steroid anti-inflammatory drugs (NSAID). Prostaglandins like PGE_2 and PGD_2 have been shown to have pro-inflammatory actions in regulating blood flow, vasodilatation and permeability of the endothelium to help leukocytes undergo adhesion and diapedesis to the site of inflammation [29]. Along these lines, leukotrienes contribute to the recruitment of neutrophils toward the site of inflammation to mediate pathogen killing. Once inflammation is initiated, inflammatory events are amplified in a cascade loop until the infection/injury is contained via immune reactions. Therefore it is crucial for the body to stop pro-inflammatory signals and start resolution mechanisms. To do so, PGE_2 and PGD_2 actively switch the transcription of enzymes required for the generation of lipoxins, resolvins and protectins (Fig.1a) [30]. The function of the lipoxins is to promote resolution of inflammation by reducing PMN infiltration [31], to stimulate nonphologistic monocyte recruitement that seems to be required for wound healing [32] and to promote the removal of apoptotic PMNs by macrophages [33].

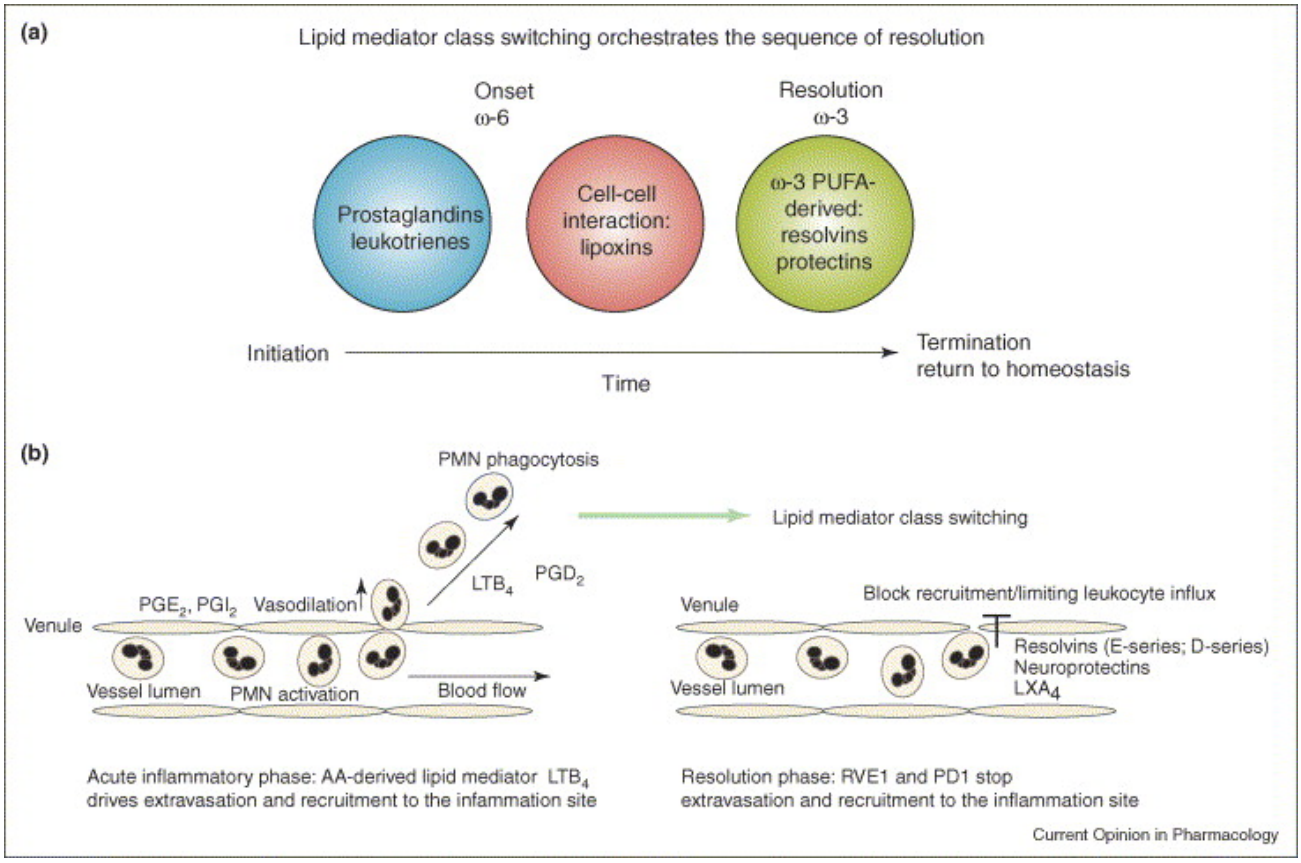

Fig.1: Switch of lipid mediators over time in inflammation [34]

(a) Sequence of lipid-derived mediators modulating acute inflammation from onset (initiation) towards resolution (termination, return to homeostasis). (b) Lipid mediators controlling influx into the inflammation site: the pro-inflammatory mediators PGE_2, PGI_2 and leukotriene LTB_4 increase the further recruitement of leukocytes at the inflammation site and with time, a class-shift occurs towards pro-resolving mediators LXA_4, RvE_1 and PD_1 that block leukocyte extravasation into the inflammation site.

1.2.2 Biosynthesis of Leukotriene B_4 and the cysteinyl leukotriene LTC_4, LTD_4 and LTE_4

The capacity to generate large amounts of leukotrienes from AA is largely limited to leukocytes. The leukotrienes are the products of the 5 lipoxygenase (5LOX), which is expressed predominantly by PMNs, mast cells, B-lymphocytes, dendritic cells and macrophages. Initially, cells can be activated by immune complexes, bacterial peptides and other stimuli, which elicit the translocation of PLA_2 to the nucleus. The primary step in the synthesis of leukotrienes is the cleavage of the arachidonyl ester bound in membrane glycerophospholipids through the action of the cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$). $cPLA_2\alpha$ is fully activated by submicromolar concentrations of calcium (Ca_{2+}) [35] and releases free AA in a hydrolysis reaction. The importance of this enzyme was demonstrated in cells deficient in $cPLA_2\alpha$ that showed an almost complete inability to

synthesize prostaglandins or leukotrienes in response to a variety of stimuli [36]. Once released, free AA has different fates and can be presented to different enzymes. Among these enzymes are the cyclooxygenase 1 and 2 (COX_1, COX_2), which metabolize AA into the intermediate prostaglandin H_2 (PGH_2). PGH_2 is in turn converted into the others prostaglandins; PGD_2, PGE_2, PGF_2 and PGI_2. Another enzyme which can transform AA is the 5LOX, which is expressed only in cells of myeloid origin [37]. 5LOX has a NH2-terminal domain that binds two calcium ions in response to increased concentrations of Ca_{2+}. The large catalytic domain of 5LOX contains an iron ion that is essential for the initial hydrogen abstraction process. 5LOX not only catalyzes the conversion of AA to 5-HPETE but also the following step to the formation of epoxide leukotriene A_4 (LTA_4). 5LOX is assisted by the five lipoxygenase activating protein (FLAP) that enhances the ability of 5LOX to interact with its substrate. Although FLAP does not have enzymatic activity by itself, it was found to be expressed in cells that synthesized leukotrienes but not cells lacking 5LOX [38]. LTA_4 is then transferred by intercellular mechanisms to different cells, which determine what kind of pathways is used to transform LTA_4. For instance, during the interaction of the endothelial cells with the PMNs, the leukotriene C_4 synthase (LTC_4S) catalyzes LTA_4 conjugation with glutathione to form LTC_4 [39, 40]. When platelets are incubated with LTA_4 they participate in LTC_4 biosynthesis [40, 41]. Once exported out of the cells, LTC_4 is subjected to extracellular metabolism forming leukotriene D_4 (LTD_4) which undergoes conversion to leukotriene E_4 (LTE_4) by sequential amino acid hydrolysis. In another pathway, LTA_4 is taken up by erythrocytes or alveolar macrophages and is hydrolytically attacked by the LTA_4 hydrolase to produce leukotriene B_4 (LTB_4) [42-44]. To perform their particular function, leukotrienes act through specific G-protein coupled receptors, which are members of the rhodopsin family and are located on the outer plasma membrane of inflammatory cells. Mostly they elicit changes in intracellular calcium and cyclic AMP concentrations which have an effect on downstream kinase cascades and lead to pro-inflammatory actions. There are two subtypes of cysteinyl leukotriene receptors, $CysLT_1$ and $CysLT_2$ that mediate actions of LTC_4 and LTD_4. $CysLT_1$ and $CysLT_2$ are expressed in airway SMCs [45, 46] where they promote bronchoconstriction, edema and mucus secretion. LTB_4 binds to specific receptors BLT_1 and BLT_2 that colocalized with markers for macrophages, endothelial cells and vascular SMCs in human carotid artery atherosclerotic plaques [47]. In monocytic cells, LTB_4 increases IL-6, MCP-1 and TNFα mRNA expression via a NF-kB-dependent mechanism, which promote pro-inflammatory and chemoattractant actions [48].

1.2.3 Biosynthesis of Lipoxin A4

Lipoxins are produced locally at sites of inflammation. The lipoxin series were first isolated from human leukocytes and were found to be conserved across species [49, 50], indicating a high biological significance across evolution. Typically, lipoxins are generated by a transcellular route. Interaction of PMNs with platelets or resident tissue cells such as epithelial cells is a good illustration of such a cell-cell interaction and demonstrates the signalling complexity of lipoxin formation. [51]. As for leukotrienes, AA is their precursor and the combined action of two or more lipoxygenases, the 5LOX and 15-lypoxygenase (15LOX) or 12-lypoxygenase (12LOX) produce lipoxins by one of at least three biosynthetic pathways. Interestingly biosynthesis of lipoxins concomitantly blocks the formation of leukotrienes at the 5LOX level [52], leading to an opposite regulation of lipoxin and leukotriene biosynthesis (Fig.1b).

1.2.3.1 15LOX pathway

The first described biosynthetic route for the generation of lipoxins involves insertion of molecular oxygen into carbon (C) 15 position of arachidonic acid by the 15LOX to generate 15S-HETE, which can be stored in lipid membranes (Fig.2) [53].

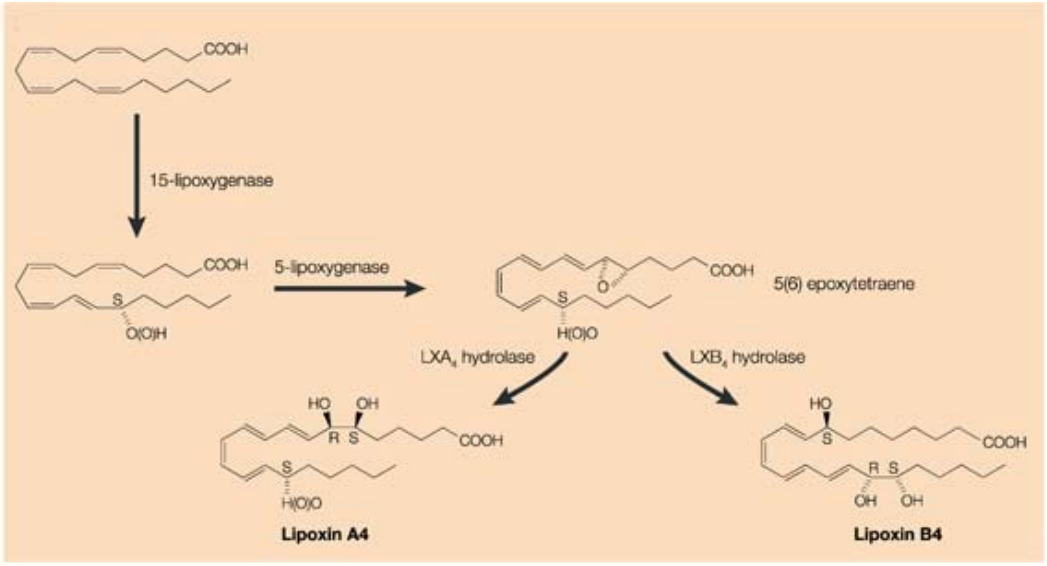

Fig.2: 15LOX pathway of the lipoxin biosynthesis [53]

In monocytes or epithelial-cell, 15-LOX transforms AA into 15 S-HETE which can then serve as a substrate for neighbouring leukocytes. 5-LOX generates 5,6-epoxytetraene which is converted to LXA_4 and LXB_4 by hydrolases.

Along these lines during the course of inflammation, activation of the cells release 15S-HETE, a product that inhibits PMN migration across cytokine-activated endothelium in vitro by reducing LTB_4 triggered stimuli [54]. Neighbouring leukocytes take up 15S-HETE, which is transformed into the 5(6) epoxytetraene. The action of specific hydrolases rapidly converts this intermediate in lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4) that are released in the micro-environnement to complete their anti-inflammatory functions [55].

1.2.3.2 5LOX pathway:

The second source of lipoxins comes from the vasculature, mainly from the interaction between PMNs and platelets. Oxygenation of AA at the carbon 5 position by the 5LOX in PMNs leads to the formation of 5-HpETE which is rapidly converted into LTA_4 (Fig.3). It appears that unprimed PMNs do not generate lipoxins on theirs own and biosynthesis of lipoxins by this route needs the interactions with other cells. About 50% of the LTA_4 is released by PMNs and could be taken up by human platelets to form LXA_4 and LXB_4 [56, 57]. The molecular profile of cell-cell interaction is of primary importance for lipoxin formation because transcellular biosynthesis needs cells adhesion. Indeed mice deficient in the adhesion molecule P selectin have a reduced efficiency of transcellular lipoxin generations, which was restored with infusion of wild-type platelets in a model of glomerular nephritis [58, 59].

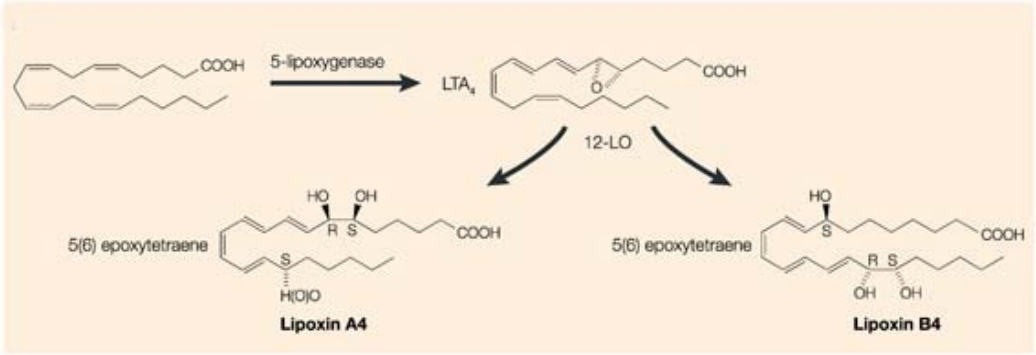

Fig.3: 5LOX pathway of the lipoxin biosynthesis [53].

5-LOX in PMNs metabolizes AA to LTB_4, which is taken up by platelets in a transcellular manner and converted by platelet 12-LOX to LXA_4.

1.2.3.3 Aspirin-triggered lipid (ATLs) pathway:

The third pathway involves the covalent acetylation of a serine residue on COX_2 by aspirin, which inhibits prostaglandin formation. The acetylated COX_2 converts AA in endothelial

cells into 15R-HETE, a metabolite that has the C15 alcohol in R configuration (Fig.4). This is due to an unusual L-shaped binding of AA with COX_2 [60]. Interaction of this intermediate metabolite with PMNs leads to the formation of 15-epi-LXA_4 and 15-epi-LXB_4 resulting from the action of 5LOX. 15-epi- LXB_4 seems to be an inhibitor of cell proliferation. Moreover in healthy individuals 15-epi-LXA_4 is produced when low dose of aspirin is taken [61] and has been proven to be a more potent inhibitor of neutrophils adhesion than LXA_4 [62].

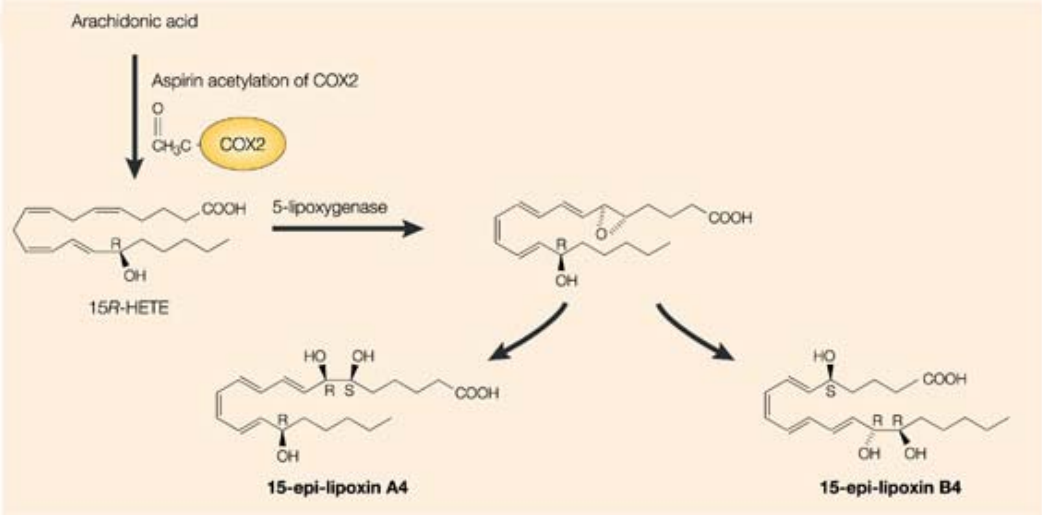

Fig.4: Aspirin-triggered lipid pathway of the lipoxin biosynthesis [53]

Aspirin acetylates the active site of COX2 resulting in the conversion of AA to 15R-HETE which when released from endothelial and epithelial cells is transformed by PMN 5-LOX to generate 15-epi-LXA_4 or 15-epi-LXB_4.

1.2.4 Lipoxin A_4 Bioaction:

Native LXA_4 is sensitive to metabolism by prostaglandin dehydrogenase and is rapidly inactivated. In contrast, the epi-lipoxins generated via the action of aspirin or synthetically produced (LXA_4 analogues) are more stable [63] and therefore have been widely used for elucidating their bioactions. In general lipoxins block the production and action of LTB_4 and LTC_4 in human leukocytes [64, 65].

More precisely, one of the main functions of lipoxins is to inhibit PMNs recruitment, chemotaxis and transmigration across the endothelium which are hallmarks of the anti-inflammatory process. Indeed PMN transmigration across polarized epithelial cell monolayers, as well as adhesion to vascular endothelial cells, was inhibited by different types of LXA_4 analogues [63]. LXA_4 and LXB_4 block PMN migration by inhibiting β2 integrin-dependent PMN adhesion and P-selectin mobilisation from the Weibel-Palade bodies, which mediates PMN endothelial cell adhesion [66]. Furthermore, LXA_4 analogues reduce leukocyte rolling and adherence by attenuating

P-selectin expression [67]. LXA_4 chemotaxis function was also confirmed in animals. Despite excessive PMN recruitment in BLT_1 transgenic mice, intravenous injection of ATLs sharply diminished reperfusion-initiated PMN trafficking. Furthermore topical application of lipoxins was protective in a model of acute dermal inflammation [68] and markedly inhibited PMNs infiltration in mouse ears [69, 70] and murine air pouches [71]. Serhan and coworkers also generated 15LOX transgenic rabbits and subjected them to periodontitis. In this study, topical application of LXA_4 stable analogues dramatically reduced leukocyte infiltration and inflammation [72]. Moreover in rats LXA_4 analogues dramatically blocked allergic pleural eosinophil influx with both inhibition of eotaxin and IL-5 generation as well as platelet activating factor [73]. Finally, administration of a synthetic cannabinoid in vitro to human blood and synovial cells increased LXA_4 production and resulted in a 25 to 75% reduction of cells invading the peritoneum in a mouse model of peritonitis [74].

LXA_4 reduces leukocytes chemotaxis in particular through regulation of chemokines. For example, in fibroblast and in pulmonary microvascular endothelial cells (PMVEC), LXA_4 inhibits IL-8 expression [75, 76] as well as IL-8 secretion in a model of *Salmonella typhimurium* infected T84 intestinal epithelial cells [77]. IL-8 is a strong chemoattractant for leukocytes and is heightened in severe asthma, where it increases eosinophil recruitment. Results from two groups of patients with asthma revealed that LXA_4 and LXB_4 inhibit the IL-8 release by peripheral blood mononuclear cells [78]. Furthermore the administration of LXA_4 analogues blocked both airway hyper-responsiveness and pulmonary inflammation by decreasing IL-5 and cyteinyl leukotrienes [79], which are both chemoattractants for eosinophils. LXA_4 not only reduces chemokine but also cytokine expression. Indeed LXA_4 prevents the elevation of pro-inflammatory cytokines such as IL-12 and IFNγ in animals exposed to T.gondii [80] and reduces both L-selectin and CD11/CD18 expression in mouse dermatitis induced by LTB_4 [81].

Another function of LXA_4 is the inhibition of superoxide production. Superoxides are byproduct of the mitochondrial respiration and are used by the immune system to kill invading microorganisms. Inhibition of their production has been shown in several cell types, for example, addition of 1-10nM of LXA_4 and ATL analogues inhibit TNFα–stimulated superoxide anion production and IL-1β release by human leukocytes [71]. Furthermore higher concentrations of ATL (1-100nM) completely blocked reactive oxygen species production in endothelial cells [82]. In PMN, 15-epi LXA_4 reversed LTB_4-initiated presqualene diphosphate production that regulates superoxide anion generation [83, 84] and selectively downregulated PMN release of azurophilic granulae [85]. Similarly in monocytes and RAW264.7 macrophages, LXA_4 analogues markedly reduced LPS-stimulated superoxide formation. This process was accompanied by an attenuation of

nuclear accumulation of AP1 and NF-kB, two transcription factor critical for vascular events [84, 86].

Another main function of LXA_4 is to stimulate phagocytosis of apoptotic PMN by macrophages. This observation was associated with actin rearrangement in THP-1 cells [87] and with an increased production of TGFβ, a negative regulator of immunity [88]. In mouse models of acute lung injury, 15-epi-LXA_4 accelerated the resolution of PMN-dependent pulmonary inflammation through redirecting PMNs to caspase-mediated cell death and facilitating their removal by macrophages [89].

1.3 **Lipoxin A_4 specific receptor**

1.3.1 Molecular characteristic of ALXR

Twenty years ago the receptor for the lipoxin A_4 (ALXR) was considered a formyl peptide receptor (FPR1) because of its DNA sequence homology and thus was named FPRL1 [90] FPRH1 [91] or FPR2 [92]. Indeed the cDNA of FPR2 was cloned from HL-60 neutrophils and its sequence shares 69% of identity with the FPR1 [90]. Since then, another gene that can cross-hybridized under high stringency conditions with both FPR1 and FPR2 was isolated and named FPR3 which shares 56% of sequence with FPR1. All of them were mapped using a panel of somatic cell hybrids to the human chromosome 19 (19q13.3-19q13.4) [91] in a cluster that span 80kb, suggesting that they may have related biological functions and may share pharmacological properties. The human FPR2 mRNA is 2.1kb long and was cloned in several types of cells including monocytes [93] and T-cells [94], as well as resident cells such as macrophages, synovial fibroblasts [75] and intestinal epithelial cells [95]. The 351 deduced amino acid sequences indicated that FPR2 belongs to the class A rhodopsin GPCR superfamily (www.iuphar-db.org) characterized by seven putative transmembrane segments with N terminus on the extracellular side of the membrane and the C terminus on the intracellular side [93, 96, 97]. The rhodopsin superfamily contains the largest number of receptors that are targeted by clinical drugs [98] and binds a great number of ligands such as peptides and lipid-like compounds. The superfamily is divided into four groups (α, β, γ, δ) and the FPR members belong to the chemoattractant receptors of the γ group which includes receptors for leukotrienes, chemokines and thromboxanes. Protein sequence alignment shows that the first and second transmembrane domains are the highest conserved regions between FPR1, FPR2 and FPR3, followed by the seven transmembrane domains (Fig.5) [99].

Fig.5: Alignement of the protein sequences of the human FPRs [99]

The putative transmembrane domains I to VII are shaded. Comparison of the three receptors has identified highly conserved regions, including most of TM-I and TM-II, and the short intracellular loop connecting TM-I and TM-II, followed by the TM-VII. The second intracellular loops from these receptors, known for G protein interaction, are also nearly identical.

Usually, extracelullar and transmembranes domains are important for ligand binding while intracellular domains are important for signal transduction and for feedback modulation of receptor function [100]. Among the FPR family, the second intracellular domain, known for G protein interaction, is identical whereas the extracellular loops differ between the receptors, suggesting difference in the nature of ligands that bind each receptor. Furthermore, the stretch of serine and threonines on the C terminal is conserved indicating that all receptors are desensitized via phosphorylation [101]. N-formyl peptide derived from bacteria and mitochondrial were identified in the mid 80's to be the shortest peptides that initiate phagocyte functions [102, 103]. The first agonist identified for FPR2 was the synthetic N-formyl-MET-LEU-PHE (fMLP) peptide. However, this ligand only binds with low affinity to the FPR2 with a Kd of 1μM [92] whereas LXA_4 binds to FPR2 with a Kd of 1.7nM [97]. Furthermore Fiore and coworker demonstrate that ^3H LXA_4 binds to PMN with a Kd of 0.5 nM [104]. Together these results suggest that LXA_4 is a specific ligand of FPR2. Thus in an effort of simplicity and to avoid any confusion ALXR will be the name used in this thesis.

ALXR orthologs have been identified in other primates [105], rats [106] and mice [107]. Mouse Fpr-rs1 and Fpr-rs2 are structurally similar to human ALXR [108] and the overall identity was found to be 76% on the nucleotide and 73% on the amino acid levels [69], which revealed good conservation of this gene between species. The gene product of Fpr-rs2 responds to several peptide agonists that activate human ALXR [109, 110]. Furthermore, the mouse Fpr-rs1 displays specific ^3H LXA_4 binding when expressed in CHO cells and initiates LXA_4 mediated GTPase activity [69].

Thirty years ago it was found that PMN responses to formyl peptide was inhibited by pertussis toxin which ADP-ribosylates the G inhibitory (Gαi) class of heterotrimeric G proteins [111]. Like formyl peptide, LXA_4 stimulates both GTPase and the release of esterifed AA in a pertussis-toxin-sensitive manner [112]. Taken together these results indicate that ALXR interacts with the G protein Gαi to mediate its specific actions.

1.3.2 ALXR regulation

1.3.2.1 Transcriptional regulation of ALXR

The transcriptional regulation of ALXR and the presence of splice variants are poorly described, since the promoter has not yet been characterized. However some regulators such as cytokines which control the expression of the ALXR mRNA have been identified. Gronert and coworkers have shown that the pro-inflammatory IFNγ and the anti-inflammatory IL-13 cytokines are the most potent inducers of ALXR expression in enterocyte (T84 cells). After 24h exposure, they increased ALXR mRNA 6,8 and 8.6 fold, and after 48h by 17.3 and 8.4 fold, respectively [95]. IL1β and LPS were the least potent pro-inflammatory cytokines, inducing a 1.8 fold increase in ALXR mRNA whereas exposure to the anti-inflammatory IL-4 or IL-6 cytokines increased receptor messenger levels by 3 fold. ALXR, therefore, can be regulated by pro and anti-inflammatory stimuli in those enterocyte cell lines depending on the micro-environment of the receptor. In synovial fibroblasts treatment with TNF-α and IL-1β was shown to up-regulate ALXR mRNA expression in a time-dependent manner whereas IL-6 had little effect on ALXR mRNA levels in these cells [113]. An inverse correlation was also found between ALXR expression and TXA_2 signaling in a mouse model of lung fibrosis and in mouse macrophages [114]. Such an anti-inflammatory character of the receptor was also described in independent studies of other laboratories. For example the glucocorticoid hormone dexamethasone has been shown to up-regulate the expression of ALXR mRNA in human PMNs in a dose-dependant manner. In contrast, the glucocorticoid receptor antagonist mifepristone suppressed the induction of ALXR mRNA by dexamethasone, indicating that the signaling via glucocorticoid receptor plays a direct role in PMNs [81]. In a parallel study, incubation of human monocytes with dexamethasone or other synthetic glucocorticoids induced the *de novo* synthesis of ALXR, leading to an increase in mRNA levels after 4–6 hours and protein levels after 12–24 hours [115]. This transcriptional regulation demonstrates that the ALXR expression responds to anti-inflammatory stimuli and may have an essential and pro-resolving role during the course of inflammation.

1.3.2.2 Post-transcriptional regulation of ALXR

GPCRs are regulated and exist in a equilibrium between two states, an inactivated (R) and an activated (G) [116]. Full agonist binding induces active conformation of the receptor which then couple with G proteins that transmit signals further to effector proteins in the cell. Negative regulation of the GPCRs exists to dampen cellular responses to the agonist and are referred as desensitization, sequestration and recycling or degradation of the receptor [100]. Desensitization is initiated by phosphorylation of the receptor by G-protein-coupled receptor kinases. Homologous desensitization was observed for ALXR as it is phosphorylated in a agonist dependent manner on the C terminal serine and tyrosine [117]. ALXR is capable of heterologous desensitization of other receptors like CCR5 and CXCR4 [118, 119] and does attenuate the cell response to their specific chemokines. Furthermore ALXR dephosphorylated the platelet-derived growth factor receptor beta (PDGFRbeta) via SH2 domain-containing tyrosine phosphatase-2 (SHP-2) to promote anti-inflammatory action on renal inflammation [120]. Usually receptor phosphorylation is followed by binding of the arrestins to uncouple the receptor with G proteins and to internalize the receptor into clathrin-coated vesicles. ALXR was shown to colocalize with β arrestin in HEK cells stimulated with the synthetic agonist peptide WKYMVM. Mouse embryonic fibroblast that lack β arrestin compromised ALXR internalisation [121]. Moreover, ALXR needs the large GTPase dynamin to be endocytosed which suggests a clathrin dependent sequestration. ALXR co-localizes with endocytosed transferrin and the small GTPases Rab4 and Rab11 in perinuclear recycling endosomes [122], indicating a slow recycling process which would target the ALXR receptor to degradation into lysosomes [123]. Rapid recycling to the plasma membrane has not been yet observed for ALXR. Interestingly, tertiary gelatinase granules of PMNs are reported to be potential stores of ALXR which are mobilized upon stimulation by LPS and other pro-inflammatory stimuli [124, 125]. Taken together, these results suggest that post-transcriptional regulation of ALXR is an important aspect of the function of the receptor and might be critical during the course of inflammation.

1.3.3 Pro and anti-inflammatory ligand of ALXR

Ligand diversity is an important aspect of the FPR family receptors. In this regard, ALXR binding pocket may be large and flexible enough for proper contact with a variety of ligands. Results obtained from different experiments indicate that specific peptides as well as lipid mediators can both function as ligands for the same receptor. ALXR can recognize several short-

lived lipids, such as lipoxin A_4 and its stable analogues, as well as proteins and peptides, such as serum amyloid protein A (SAA) and those derived from viral glycoproteins (Fig.6). Interestingly, receptor N-glycosylation, which is important for ligand specificity, is present on two asparagines of the ALXR (Asn 4 and Asn 179) and deglycosylation reduces peptide affinity and recognition for ALXR. The property, that fMLP binds with low affinity ALXR, was used for mapping the ALXR ligand binding domain. Seven chimeric FPR1/ALXR and three chimeric ALXR/FPR1 receptors were constructed by selective substitution of one receptor's fragment into the other receptor. The results show that the first and third extracellular loops are essential for high affinity binding of fMLP [126]. Conversely chimeric receptors constructed from the receptor BLT1 and ALXR revealed that the seventh transmembrane segment and adjacent regions of ALXR are essential for LXA_4 recognition [127]. Theses two independent results suggest that ALXR has different binding pocket related to the nature of the ligand.

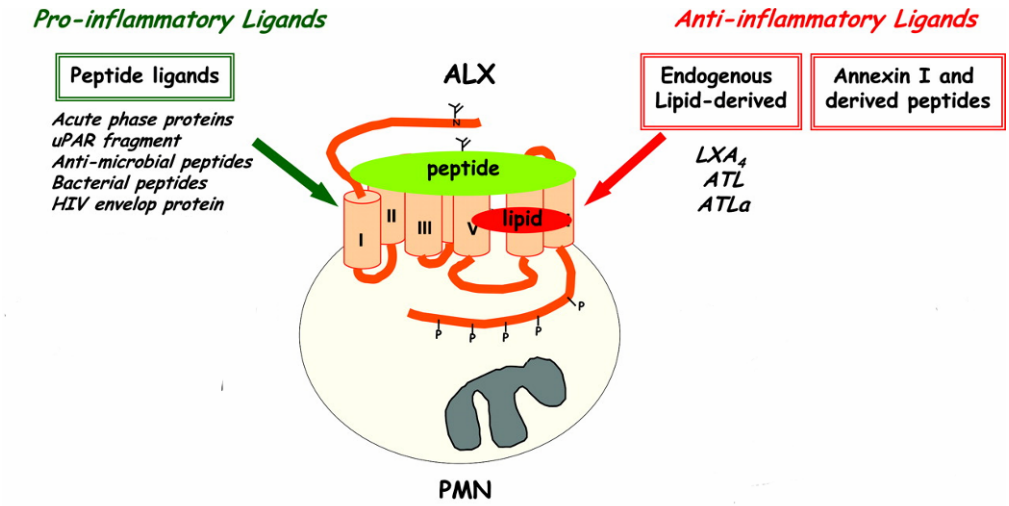

Fig.6: Pro- and anti-inflammatory ligands of ALXR [128]

Both peptide and lipid-derived ligand bind to ALXR to modulate pro- and anti-inflammatory actions.

During the pro-inflammatory phase, host response to injury and infection is accompanied by a rapid rise in the blood of acute-phase proteins like serum amyloid A (SAA). Ten years ago, SAA was one of the first pro-inflammatory chemotactic ligands identified for ALXR [129] which led to the increase of IL-8 secretion following activation of NF-kB in human PMNs [130]. SAA induced via ALXR other pro-inflammatory actions such as matrix-metalloproteinase 9 (MMP9) generation [131] and chemokine CCL2 production in a concentration-dependent manner in human monocytes [132]. Since the discovery of SAA as a pro-inflammatory ligand for ALXR, more peptide/proteins were identified as ligands for ALXR. For example, cleavage of the chemokine CCL23 by a protease present during inflammation releases a 18 amino-acid peptide (SHAAGtide) which recruits monocytes and PMNs in vitro via ALXR [133]. Moreover, bacteria-derived peptides

like Helicobacter pylori derived peptide Hp-(2-20) can activate ALXR [134] as well as synthetic peptides like MMK-1, which induces chemotaxis and calcium mobilization in leukocytes [135-137]. MMK-1 activates PMNs to produce and release superoxide anions through release of Ca_{2+} from intracellular stores and through opening of ion channels in the plasma membrane [138]. Finally, in brain tissue of neurodegenerative Alzheimer disease's patient ALXR is expressed at high levels on inflammatory cells infiltrating senile plaques and signals a chemotatic response to beta amyloid peptide (Aβ42) [139]. To conclude, these pro-inflammatory peptides/proteinS do not share apparent homology in terms of the primary amino acid sequences and further investigation of their role in human pathology has not been yet determined. However, they were shown to induce inflammatory actions through ALXR, which might therefore play a role in the pro-inflammatory phase of inflammation.

The anti-inflammatory effects of ALXR became apparent when LXA_4 was identified as an endogenous ligand for ALXR. Using freshly isolated radioligands, specific LXA_4 binding sites were characterized on isolated human PMNs and demonstrated to be responsible for the specific LXA_4 function [104]. Synthetic 15-epi-LXA_4 and LXA_4 have shared stereospecific actions for activating ALXR-regulated gene expression and for their ability to inhibit human PMN migration through endothelial cells [140, 141]. Indeed LXA_4 has been shown to decrease SAA-induced IL-8 secretion [130, 142] and 15-epi-LXA_4 inhibits VCAM and E-selectin expression in endothelial cells [143]. In addition to the inhibition of PMN chemotaxis and migration, LXA4 inhibited TNFα secretion by T cells [94], induced chemotaxis of monocytes without cytotoxicity [93] and has been shown to promote nonphlogistic phagocytosis of apoptotic PMNs by human macrophages [18, 33, 144]. The glucocorticoid-induced Annexin A1 (ANXA1) and its derived peptide Ac2-26, generated in vivo, could also halt PMN diapedesis. Both of these anti-inflammatory responders were shown to interact directly with recombinant human ALXR in human embryo kidney cells transfected with ALXR and in primary PMNs [145]. In another study Gavins and co-workers demonstrate that ANXA1 inhibited firm adhesion of human PMNs, while Ac2-26 significantly attenuated capture and rolling [146]. Recently in vivo data confirmed the anti-migratory action of ANXA1 in knock out mice and the co-localization of ALXR with ANXA1 in PMNs that migrate into the mesenteric microcirculation [147]. Thus ALXR possibly induces anti-inflammatory actions when pro-resolving mediators such as LXA4 and ANXA1 are present during the late phase of inflammation.

The dual role of ALXR as a pro-inflammatory or anti-inflammatory receptor is supported by the opposite actions of ALXR during HIV infection. In one way ALXR is a co-receptor for HIV/SIV [148] helping HIV to target and enter cells but in another way a synthetic peptide domain of the V3 region of the HIV-1 envelope gp120 activates ALXR in monocytes [149] which resulted

in the downregulation of the HIV-1 chemokine co-receptors CCR5 and CXCR4 [150], therefore blocking HIV-1 entry. Furthermore, depending on the cell types, the same ligand can induce different effects via ALXR. In fibroblast LL-37 generates superoxide generation with activation of the NADPH oxidase [151] while in PMNs, it was found to inhibit the pro-inflammatory actions of SAA via inhibition of ERK and p38 MAPK activity [152].

The finding that specific endogenous lipid mediators and certain peptides interact with the same receptor may reflect at the genomic level the economy of using one receptor structure for multiple recognitions and functions in the immune system. Furthermore, competition between these pro and anti-inflammatory agents points towards an important role of ALXR during the onset of chronic inflammatory diseases and its resolution.

1.3.4 LXA_4-ALXR downstream signaling

The current understanding of the ALXR's signal transduction pathways remains incomplete and there are no clear mechanisms of the pathway that explain the pro- and anti-inflammatory functions of the receptor. Receptor dimerization upon ligand binding could be observed for leukotriene and chemokine receptors but no study reported ALXR homo or hetero dimerization [153, 154]. GPCRs activation induces guanosine diphosphate GDP release (inactive state) and guanosine triphosphate GTP binding (active state) to a heterotrimeric G protein that is dissociated into Gβγ protein and Gα protein. Gα protein is the effector protein that possesses GTPase activity and transmits downstream signals in the cells. LXA_4 binding to ALXR was shown to stimulate GTPase activity [112] and to increase the intracellular content of Ca_{2+} followed by the activation of phospholipase A_2 and phospholipase D [93]. All of these responses were shown to be sensible to the treatment with the specific Gi inhibitor PTX which stops the production of cyclic adenosine monophosphate (cAMP) from ATP by decreasing adenyl cyclase activity. This enzyme induces normally cAMP dependent protein kinase A which phosphorylates other proteins and is important for cell cycle and carbohydrate and lipid metabolism.

LXA_4 binding to ALXR has been shown to promote monocyte chemotaxis without increasing cell-mediated cytotoxicity and reactive oxygen generation. These effects are typically anti-inflammatory actions that are needed to reduce the pro-inflammatory state and to restore tissue homeostasis. An interesting study investigated the downstream signaling of ALXR when ATL1 are used to induce human monocyte chemotaxis. ATL1 bound to ALXR activated the Gαi protein that triggered Ras homolog gene kinase dependent pathway (Fig.7). The Rho kinases induce phosphorylation of the extracellular signal-regulated kinases (ERK-2), a member of the mitogen-

activated protein kinase (MAPK) family. This observation was consistent with previous studies which showed that distinct peptide ligands increased ERK-2 phosphorylation through the activation of ALXR [132, 155]. In this study ATL1 also increased myosin light chain kinase (MLCK) phosphorylation in an ERK2 dependent fashion, suggesting that MLCK could be a downstream step of the MAPK pathway in monocytes. MLCK can then phosphorylate the myosin light chain (MLC), which promotes cytoskeleton contraction necessary for cell movement. A Rho kinase inhibitor Y-27632 abrogated both MLCK phosphorylation and chemotaxis stimulated by ATL-1, indicating that the Rho/Rho kinase pathway may be necessary for ATL-1-induced monocyte chemotaxis via MLCK. Therefore, Simon and co-workers hypothesized that ATL1 acting via ALXR promotes monocytes chemotaxis through polymerization of actin cables [156].

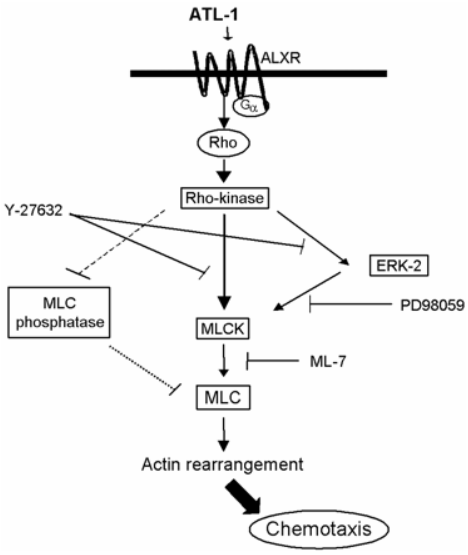

Fig 7: ALXR chemotaxis downstream signaling [156]

ATL-1 occupies ALXR, which results in the activation of the Rho/Rho-kinase pathway. Rho-kinase phosphorylates ERK-2 and MLCK, leading to the induction of MLC and actin rearrangement, which permit cell chemotaxis.

Another important function of LXA_4 is to induce the removal and phagocytosis of apoptotic PMNs by human macrophages in inflamed tissue. Cell clearance is an essential step of the resolution phase of inflammation. A recent study investigated the ALXR downstream signaling and proposed a pathway for the pro-apoptotic action of ALXR in PMNs, which are rapidly removed from the tissue. The authors suggest that LXA_4 and ATL inhibit and override the survival signals from SAA. Indeed SAA induces the PI3kinase and MEK pathways which evoke rapid phosphorylation of the protein kinase Akt and ERK1/2 respectively (Fig. 8). Both pathways activate the proapoptotic B-cell lymphoma 2 (Bcl2)-associated death promoter protein (Bad) which is dissociated from the antiapoptotic myeloid leukemia cell differentiation protein (Mcl1). Mcl-1

prevents the loss of mitochondrial transmembrane potential which normally releases cytochrome c and activates caspase 3, a activator of apoptosis [157]. Thus SAA reduces apoptosis and prolonges the survival of PMNs. These effects have been shown to be inhibited by LXA_4 and ATL-1 actions through ALXR which therefore, promotes pro-resolution mechanisms.

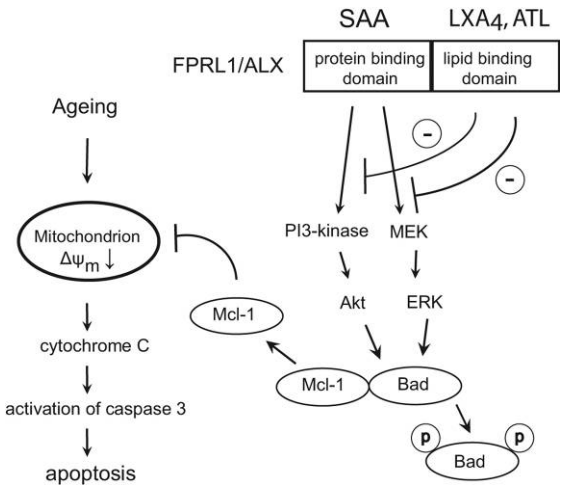

Fig.8: ALXR apoptosis downstream signaling [157].

SAA binds to the protein-binding domain of the FPRL1/ALX and induces phosphorylation of the proapoptotic protein Bad through activation of the PI-3K/Akt and MEK/ERK pathway. Phosphorylated Bad then dissociates from Mcl-1, preventing the collapse of mitochondrion $\Delta\Psi_m$, cytochrome c release, and activation of caspase-3, characteristic features of PMNs undergoing constitutive apoptosis. LXA_4/ATL binds to the lipid-binding domain of FPRL1/ALX and over-rides the SAA survival signal and redirects PMNs to apoptosis.

1.3.5 ALXR evidence in animals and human disease

Recently, an interesting study described for the first time ALXR knock out mice which were more sensitive to inflammation with a pronounced increase in cell adherence and emigration in the mesenteric circulation after ischemia-reperfusion insult. This study showed that SAA stimulated PMNs recruitment whereas LXA_4 and Ac2-26 had anti-migratory effect on the same cells, indicating that ALXR can have pro-inflammatory actions which are inhibited in the presence of strong competitors like LXA_4 and Ac2-26 [158]. The anti-inflammatory effect of the ALXR is also supported in transgenic mice that expressed human ALXR which led to a significant inhibition of pulmonary inflammation and eicosanoid-initiated eosinophil tissue infiltration [79]. Reduced PMN infiltration was also observed in mice following dermal ear inflammation [159] and acute lung injury [160]. Furthermore, in inflammatory hyporesponsiveness mice administration of a 15-epi-LXA_4 analogues or an annexin-1-derived peptid prevented tissue injury, TNFα production, and lethality after intestinal ischemia/reperfusion. This process was associated with the release of IL-10 production and was reversed by treatment of the mice with an antagonist of ALXR (BOC1) [161].

Together these data indicate that ALXR is an important modulator of the outcome of inflammation. Resolution of inflammation is mediated by extracellular stimuli and the corresponding signals that cells transmit through receptor. Thus ALXR knock out and hALXR transgenic mice are of great importance to visualize the protective role of this receptor in the anti-inflammatory and pro-resolution response relevant to human disease. ALXR is expressed in several human diseases such as dermatitis, where ALXR was found to be on membranes of PMNs which had exocytosed the content of their granules [162]. Furthermore, expression of ALXR is strong in rheumatoid arthritis [163] and in injured bronchial epithelials [164]. However, LXA_4 and ALXR generation decreased in bronchoalveolar lavage fluid obtained from subjects with severe asthma as well as in peripheral blood granulocytes, like PMNs and eosinophils [165]. Thus, ALXR and its pro- and anti-inflammatory agonist seem to be important players in inflammation and human disease and may give new approach for the therapeutic control of inflammation.

1.4 **Atherosclerosis**

Atherosclerosis is a wide-spread disease with a long asymptomatic phase and is the primary cause of cardiovascular disease CVD like coronary artery disease (CAD) leading to myocardial infarction MI (heart attack) and stroke (cerebrovascular attack) [166]. Atherosclerosis advanced lesions start at the age of 25 years and clinical manifestation occurs in 2 of 3 men and 1 in 2 women after age of 40 [167]. The common risk factors are multiple and include diabetes, cigarette smoking, dyslipidemia with high concentration of low-density lipoprotein cholesterol LDL-C and low concentration of high-density lipoprotein cholesterol HDL-C, hypertension, advanced age and family past history (www.agla.ch). Family history in atherosclerosis increases the risk of developing a cardiovascular event when the number of relatives or the age at which coronary heart disease (CHD) first becomes evident is taken into account [168].

Atherosclerosis is characterized with the formation of plaques that narrows the arteries (stenosis) and is visualized at angiography. Even though bacteria and virus-derived antigens have been found in atheroma lesions [169-171], a number of evidence suggests that when entering the arteries LDL-C are modified by enzymes and oxygen radicals into oxidized LDL (oxLDL) which is recognized as foreign agent and leads to the activation of an inflammatory response [172]. PPRs implicated in atherosclerosis are TLR4 [173] or SRs such as SR-AI and CD36 which have been found to be the most important receptors in the uptake of oxLDL by macrophages [174, 175]. Excessive amount of circulating cholesterol elicits then an inflammatory response from arterial wall tissue. Endothelial cells have been shown to express VCAM-1 in response to cholesterol feeding in areas prone to

lesion formation [176]. Other chemokines and cytokines have been shown to be implicated in atherosclerosis such as E and P-selectin which contribute to leukocytes recruitment in atherosclerosis-susceptible mice [177] or ICAM in which genetic deletion inhibits atherosclerosis in mice [178]. Signaling molecules such as IL-1β, IL-12, IL-8, IL-6 and TNFα have been associated with an increased risk of CAD and recurrent MI [6-8]. Furthermore, endothelial function is impaired during atheroslcerosis via disruption of the endothelial nitric oxide synthase (eNOS) activity (Fig.9a). As a consequence, vasorelaxant nitric oxide (NO) production is impaired [179] and might lead to the development of premature atherosclerosis [180].

For a long time it was thought that PMNs were not present in plaque lesion and do not contribute to the pathogenesis of atherosclerosis. Nevertheless, recent evidence suggest that it is not the case and PMN infiltration was found in various intermediate and advanced stages of atherosclerosis in low density lipoprotein receptor deficient (LDLR-/-) mice [181, 182], in coronary lesions obtained at autopsy from patients with myocardial infarction [183] and in patients with unstable plaques. PMN count appears to be higher in such patients than those with stable disease suggesting a link between PMN infiltration plaque fragility and haemorrhage [184]. Prolonged PMN survival has been shown to be mediated by several factors like IL-6, IL-8, TGFβ and hypoxia and their apoptosis has been shown to be delayed in acute coronary syndrome, contributing to an exponential increase of inflammation [185]. Despite this prolonged survival of PMNs, they still undergo apoptosis and are taken up by monocytes-derived macrophages. However, in atherosclerosis macrophages take up also esterified cholesterol and are transformated into foam cells [186] which is the major cellular event contributing to fatty streak formation (Fig 9b)

Transition from fatty streak to complex lesion is accompanied with the recruitment of SMCs from the media layer to the intima layer. (Fig.9c). SMCs proliferate and take up modified lipoproteins contributing to foam cell generation and collagen-derived fibrous cap formation [172]. In addition to the recruitment of SMC, angiogenesis and neovascularization are processed that serve at the site of inflammation to perform nutritive function and to promote plaque growth [187]. With the time, cells undergo programmed cell death resulting from cell-cell interaction and cytokine presence in the micro-environment. Macrophages are rapidly overloaded with apoptotic cells and lipids and release cell debris. Cells undergo into necrosis and form a lipid core that induces plaque instability [188]. Release of matrix metalloproteinase like MMP9 from macrophages degrades extracellular matrix proteins [189, 190] and pro-inflammatory cytokines like IFNγ starts to inhibit production of collagen by SMC [191].

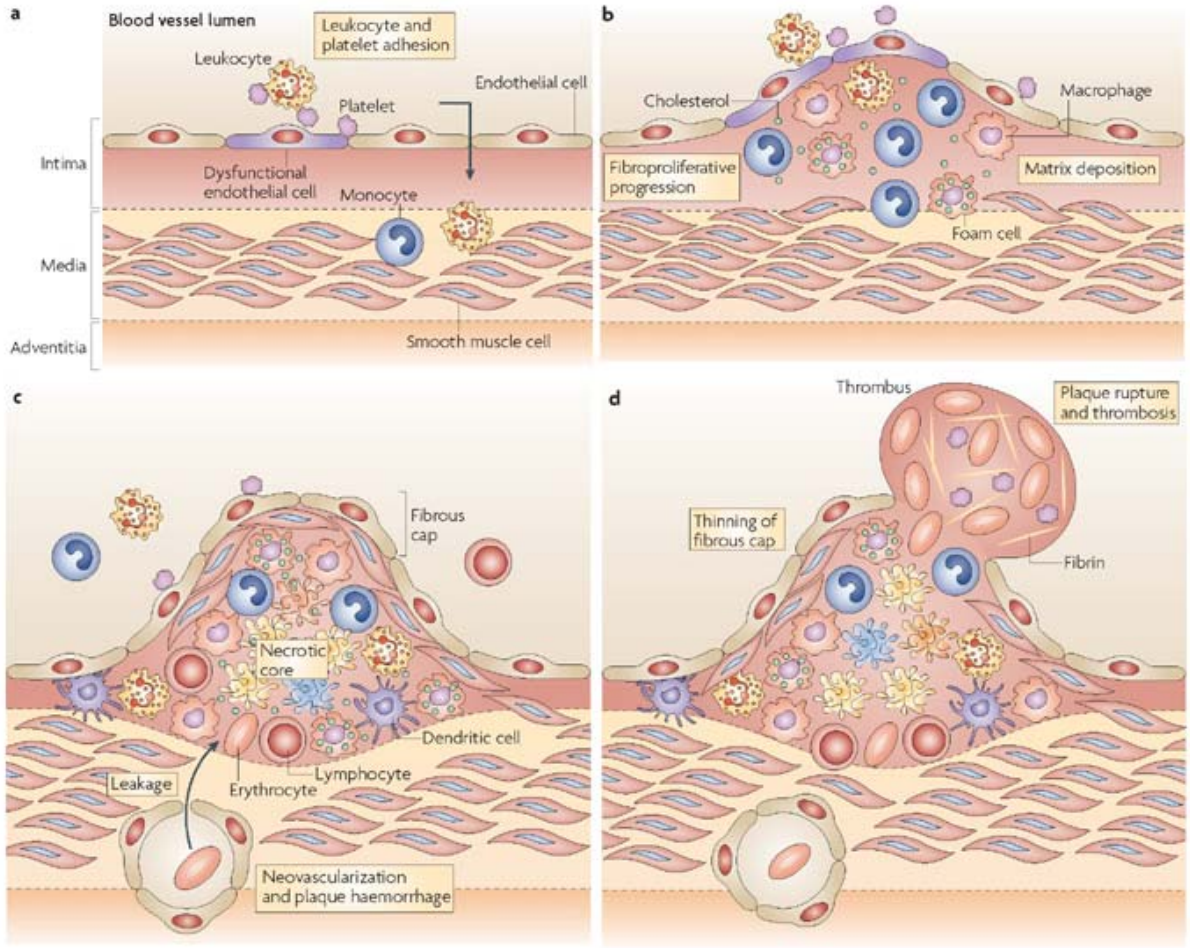

Fig.9: Evolution of atherosclerosis [192]

a. Activation of pro-inflammatory conditions leads to leukocyte adhesion and increased permeability of the endothelium. B. Monocyte-derived macrophages that are recruited to the intima accumulate lipids and transform into foam cells, which make up fatty streaks. **c.** Apoptosis of macrophages and other plaque cells create a necrotic core, and a fibrous cap that consists of matrix and a smooth-muscle-cell layer forms. Neovascularization can occur within the plaque and leakage of fragile vessels can lead to plaque haemorrhage. d. Thinning and erosion of the fibrous cap in unstable plaques because of matrix degradation by proteases, result in plaque rupture, with release of debris, activation of the coagulation system and plaque thrombosis of the artery.

Finally, advanced atherosclerotic lesions lead to narrowing of blood vessels and to ischemic symptoms. Most myocardial infarction and stroke events are due to plaque rupture and thrombosis formation (Fig 9d). Indeed, endothelial desquamation can be observed, in which von Willebrand factor proteins recruit coagulation factor like Factor VII and collagen. This will promote platelet adhesion and activation of the coagulation system like thrombin-mediated fibrin production [193]. Blot clot formation and plaque rupture prevent blood flow and leads to ischemia of the myocardial muscle. Morbidity and mortality from atherosclerosis is largely due to disruption of unstable plaque, haematoma or haemorrhage and thrombotic deposit.

1.4.1 Lipid mediator in atherosclerosis

The role of leukotrienes in atherosclerosis has been the subject of intense investigation in animals and in vitro studies in which they promote the recruitment of monocytes and differentiation into foam cell as well as intimal hyperplasia [194]. LTB_4 receptor is expressed in cells that are involved in atherosclerosis [195] and treatment of $LDLr^{-/-}$ and $apoE^{-/-}$ deficient mice with a specific LTB_4 receptor antagonist CP-105,696 for 35 days, significantly reduced the chemoattractant agent CD11B in both vascular lesions and whole blood [196]. Along these lines combination of $BLT_1^{-/-}$ and $apoE^{-/-}$ knock out mice demonstrated a clear reduction of plaque formation compared to $apoE^{-/-}$ controls [197]. Also the LTB_4 receptor genes have been intensely searched for genetic variant that would be associated with atherosclerosis. Recently haplotype in the LTB_4 receptor complex was found to confer a 2.3 fold increase in risk of ischemic stroke [198]. Human arteries subjected to angioplasty and stent implantation displayed an increased intima/media thickness and higher MMP-2 and -9 activities in the presence of LTB_4 [199]. Moreover it was found that $CysLT_1$ mediated vascular permeability and tissue fibrosis [200] and that an inhibitor of $CysLT_1$ significantly decreased atherogenesis [201]. Furthermore, enzymes leading to the production of leukotrienes have been shown to be involved in the development of atherosclerosis. For instance, 5LOX is higher expressed in vulnerable plaques [202] and has been correlated with the severity of atherosclerotic lesions [203, 204]. Polymorphisms in the 5LOX promoter were found to be associated with increased intima-media thickness of the carotid artery [205] and with coronary disease [206]. FLAP was also identified as a susceptibility gene for myocardial infarction and stroke following a genome wide screen performed in Icelandic individuals [207]. Together these results indicate that leukotrienes and the enzymes, leading to their biosynthesis, are enhancers of atherosclerosis.

It is now generally accepted that generation of anti-inflammatory lipid mediators is implicated in the resolution of inflammation. 15LOX is an enzyme expressed in eosinophils, alveolar macrophages, monocytes and epithelial cells [208, 209] and is a Janus enzyme with pro-atherogenic and anti-inflammatory properties. There is indication that the 15LOX oxidizes LDL which is taken up by macrophages to develop foam cells. Additionally, overexpression of 15LOX in $LDLr^{-/-}$ deficient mice increased lesion formation and development [210]. However, there are contradictory results concerning the pro-inflammatory effect of 15LOX. In 1995 Shen and co-worker generated a rabbit overexpressing 15LOX in macrophages, which resulted in a 45% decrease in aortic lesion area [211]. 15-LOX-deficient macrophages displayed significantly reduced levels of LXA_4, which correlated with an enhanced inflammatory gene expression [212]. In

addition, a c.-292 C>T polymorphism found in the human 15LOX promoter of macrophages and a c.1693 C>T polymorphism (T560M mutation) leading to an inactive enzyme, have been associated with CAD, suggesting that 15LOX protects from atherosclerosis [213, 214]. However, these two polymorphisms were not associated with myocardial infarction indicating a spatial and temporal effect of 15LOX in atherosclerosis[215]. Furthermore, 15LOX is implicated in the formation of the anti-inflammatory lipoxins which support the anti-atherogenic role of 15LOX. Recently, LXA_4 has been shown to be a potent modulator of angiogenesis, which can appear in the development of late phase of atherosclerosis [216]. Revascularization of the vessels is associated with lesion formation and plaque instability and needs the migration of endothelial cells to organize the formation of the vessels. Investigation of the effects of LXA_4 analogues in reorganization of endothelial cells stimulated with VEGF show an inhibition of endothelial cell migration via the concerted inhibition of actin polymerization [217]. LXA_4 also inhibits VEGF-stimulated expression of IL-6, TNFα, IFNγ and IL-8 secretion in human umbilical vein endothelial cells HUVEC [218]. In addition 10µg of ATL-1 analogues in mice reduced the angiogenic phenotype by approximately 50%, indicating that lipoxins are important for plaque stability and the onset of cardiovascular disease [219]. Taken together, these results indicate that lipoxins have anti-inflammatory and pro-resolution effects on atherosclerosis and open new opportunities for treatment of chronic diseases.

1.4.2 ALXR as potential target of atheroslcerosis

Data reported in the GISSI (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico) study demonstrate a reduction in sudden death of 45% in patients at risk for myocardial infarction for those who were taking omega 3 and aspirin every day (http://www.gissi.org/). Indeed in 2002 a meta analysis shows that because of its effective anti-platelet function 75-150mg of aspirin taken daily is protective in most patient at increased risk of occlusive vascular events, including those with an acute myocardial infarction or ischemic stroke and unstable or stable angina [220]. Aspirin blocks COX_1 and COX_2 by covalently acetylation of a serine residue, thus competing with AA for binding at the active site. Thus, the acetylation of COX_2 shifts the enzyme from producing the pro-inflammatory prostaglandin to the anti-inflammatory 15-epiLXA_4 [221] and this was also shown with low dose aspirin in humans [61]. Furthermore, newly developed dual COX-5-LOX inhibitors share the anti-inflammatory effect and gastric safety of COX_2 inhibitors, but also inhibit COX_1-mediated platelet function and 5-LOX-mediated synthesis of gastro-toxic leukotrienes [222].

Interestingly for ALXR, a recent study used computational biology approach to predict novel GPCR peptide ligands that are cleaved from secreted proteins by convertase proteolysis. These ligands were screened for their calcium flux and cAMP activities. The CGEN-855A ligand is a novel agonist for ALXR and displays anti-inflammatory activity manifested as a 50% inhibition of PMN recruitment to zymosan induced inflamed air pouch. More interesting, CGEN-855A reduced 36% and 25% of the infarct size in murine and rat models of ischemia-reperfusion-mediated injury to the myocardium. The secretion of inflammatory cytokines, including interleukin IL-6, IL-1β, and TNFα, were not affected upon incubation of human peripheral blood mononuclear cells with CGEN-855A, whereas IL-8 secretion was elevated up to 2-fold upon treatment with the highest CGEN-855A dose only [223]. CGEN-855A is therefore an interesting therapeutic drug that binds ALXR and could be used to treat acute and chronic inflammation.

2 RESULTS

2.1 **Manuscript**

Characterization of the promoter and transcriptional regulation of the ALXR gene in human monocytes and macrophages.

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Contribution:

1. Performed the experiments and generated the figures:
   Figure 1 A/B/C
   Figure 2 A/C; Figure 2 B/D in collaboration with Viola Günther (Group Prof. Schaffner)
   Figure 3 A/B/C in collaboration with the master student Angelika Weber
   Figure 4 A/B/C; Figure 4 D in collaboration with Viola Günther (Group Prof. Schaffner)
   Suplementary data Table S1/S2/S3/S4

2. Wrote the manuscript
   Abstract; Introduction; Experimental procedure, Results, Discussion, Figure legends

Characterization of the promoter and the transcriptional regulation of the *ALXR* gene in human monocytes and macrophages

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Abstract

The lipoxin A4 receptor (ALXR) plays an important role in host defense and inflammation. The receptor binds structurally diverse agonistic ligands, including peptides and lipid mediators, which mainly regulate chemotaxis and activation of leukocytes. While the effects of these ligands in leukocytes have been investigated, little is known about the promoter region of the *ALXR* gene and about its transcriptional regulation in leukocytes. We identified two TATA-less promoter regions separated by 224 bps which drive the expression of the ALXR in macrophages. Both promoter regions increased transcription in a reporter assay and the basal transcription factors, OCT1 and SP1, were shown to bind the first and the second promoter, respectively, and to transactivate transcription. To investigate the ALXR mRNA expression in monocytes and macrophages, we measured basal expression of ALXR mRNA during differentiation of monocytes to macrophages and stimulated mRNA expression in macrophages. While monocytes expressed high levels of ALXR mRNA, differentiation into macrophages abrogated ALXR expression. Stimulation of macrophages with a set of cytokines revealed that only IFNγ increased ALXR expression to levels similar to the ones detected in monocytes. This upregulation by IFNγ is in part mediated by IRF1 interacting with an IRSE transcription factor binding site located in the first promoter region of the *ALXR* gene. These data support the model of ALXR playing a role in chemotaxis and activation of monocytes, however, they also suggest that its role in macrophages is limited to activated macrophages stimulated with IFNγ.

Introduction

The lipoxin A4 receptor (ALXR) belongs to the class A rhodopsin G protein-coupled receptor superfamily (1) and plays a role in chemotaxis and activation of phagocytes (2). Several structurally diverse agonistic ligands, including peptides and lipid mediators have been shown to bind to the ALXR. Serum amyloid A (SAA) was one of the first chemotactic ligands identified for ALXR (3) which led to an increase of IL-8 secretion in human polymorphonuclear neutrophils (PMNs) (4). SAA also induced matrix-metalloproteinase 9 (MMP9) generation (5) and CCL2 production in a concentration-dependent manner in human monocytes (6). Since the discovery of SAA as a pro-inflammatory ligand for ALXR, more peptides were identified that bind ALXR such as the SHAAGtide cleaved from the chemokine CCL23, the Helicobacter pylori derived peptide Hp-(2-20), the synthetic peptide MMK and the beta amyloid peptide (Aβ42)(7-14). These peptides do not share apparent homology in terms of the primary amino acid sequence but induce all pro-inflammatory activation of leukocytes through ALXR (2).

ALXR can also bind anti-inflammatory ligands such as LXA_4 (15) and annexin-1 (16) which leads to reduced PMN recruitment into inflamed tissue. LXA_4 was shown to inhibit PMN migration (17), to induce chemotaxis of monocytes (18,19) and to promote the nonphlogistic phagocytosis of apoptotic PMNs by macrophages (20-22). Similarly, the glucocorticoid-induced annexin A1 (ANXA1) and its derived peptide Ac2-26, also suppressed PMN diapedesis in mouse models of inflammation through ALXR (23-26). This anti-inflammatory effect of the ALXR is further supported by findings in two genetically altered mouse models. Mice expressing the human ALXR showed a significant inhibition of

pulmonary inflammation and mice lacking the *ALXR* homologue showed increased emigration of leukocytes after ischemia-reperfusion injury (27) Thus ALXR might induce anti-inflammatory actions when pro-resolving mediators such as LXA4 and ANXA1 are present.

These opposing effects mediated by the ALXR upon binding of pro- and anti-inflammatory ligands, suggests that ALXR expression is restricted to certain cell types and that its expression is tightly regulated. Indeed ALXR mRNA is expressed in a limited number of primary cells involved in inflammation such as, monocytes (18), neutrophils (28), NK cells (29), and possibly T-cells (17,30). However, regulation of ALXR expression is not well understood, with a limited number of regulators identified. In synovial fibroblasts treatment with the pro-inflammatory TNF-α and IL-1β was shown to up-regulate ALXR mRNA expression in a time-dependent manner, whereas IL-6 had little effect (31). In enterocytes, the pro-inflammatory cytokine IFNγ and the anti-inflammatory cytokine IL-13 were shown to be the most potent inducers of ALXR expression, while IL1-β and LPS only slightly increased the expression (32). In mouse macrophages an inverse correlation was found between ALXR expression and TXA_2 signaling (33). In NK cells, IL-10 stimulation led to a moderate induction of ALXR mRNA (29), and the glucocorticoid hormone dexamethasone increased the expression of ALXR mRNA in a time- and dose-dependent manner in PMNs (34,35). These findings indicate that ALXR may be regulated by pro and anti-inflammatory stimuli and that the response to stimulation depends on the cell type investigated.

Since little is known about the promoter region of the *ALXR* and about its transcriptional regulation in leukocytes, we set out to define the basal promoter of the *ALXR* gene and to investigate its transcriptional regulation in human macrophages. We located two TATA-less promoter regions in which the basal transcription factors OCT1 and SP1 were found to be necessary for ALXR transcription, respectively. We further show in primary human cells that monocytes express high levels of ALXR mRNA, while differentiation into macrophages abrogated this expression. IFNγ stimulation of macrophages rescued ALXR mRNA expression in a dose-dependent manner, an effect which was at least partially mediated by IRF1 via an IRSE element located in the first promoter of *ALXR*. These data support the model that the ALXR plays a role in chemotaxis and activation of monocytes while its role in macrophages seems restricted to activated macrophages stimulated with IFNγ.

Experimental procedures

Material

The recombinant human cytokines r-interleukin 13 (IL-13), r-interleukin 1 beta (IL-1β), r-tumor necrosis factor alpha (TNFα), r-transforming growth factor beta (TGFβ) and r-interferon gamma (IFNγ) were purchased from Sigma-Aldrich (Buchs, Switzerland)

Primary cell purification and cell culture

Leukocytes from healthy volunteers were extracted from 60 ml of heparinized blood or buffy coat (Blutspendezentrum Zürich, Switzerland) with Histopaque-1077 gradient (Sigma-Aldrich). Peripheral blood monocytes were purified by capturing with anti-CD14 antibodies coupled to MACS Microbeads (Miltenyi Biotec, Bergisch Glad, Switzerland) according to the manufacturer's instructions. Monocytes were allowed to differentiate into macrophages for 7 days at 37°C in RPMI-1640 (Sigma-Aldrich) supplemented with 5 % FCS (Bioconcept, Allschwil, Switzerland), 5 % human AB serum (Sigma-Aldrich), 1% Penicillin/Streptomycin (Invitrogen, Basel, Switzerland) and 5 % CO_2. Human THP-1 monocytes (36) were cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10 % Fetal Calf Serum (FCS) (Bioconcept), 20 mM glutamine (Invitrogen) and 5 % CO_2. For differentiation into macrophages, cells were stimulated for two days with phorbol-12-myristate-13-acetate (Sigma-Aldrich) (37).

Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from THP-1 and primary macrophages using TRI Reagent (Molecular Research Center, Cincinnatti, USA). 5'RACE was performed using the RLM-RACE kit (Ambion, Rotkreuz, Switzerland) according to the manufacturer's instructions. cDNA synthesis was performed with random decamers and MMLV reverse transcriptase. The first PCR reaction was performed with the kit's 5'race outer primer and a reverse primer for ALXR (5'CCACCACGATGTGAATTAACT3'), designed with the OLIGO 6.0 software (Medprobe; www.medprobe.com) on the sequence NM001005738.1. The nested PCR was carried out with the 5'RACE Inner primer and the nested reverse primer for ALXR (5'TGGTAATGTGGCCGTGAAAGAAAA3').

The PCR products were loaded on 2 % agarose gel and the corresponding bands were eluted from the gel and sequenced.

Quantification of ALXR mRNA

The RT reaction was performed on 1 µg total RNA with Superscript III reverse transcriptase (Invitrogen) with random primer according to the manufacturer's instructions. Each RT-PCR was performed in a total volume of 20 µl on a Light Cycler system (Roche Diagnostic, Rotkreuz, Switzerland). ALXR mRNA quantification included 100 ng cDNA, 4 mM $MgCl_2$, 0.5 mM upper primer (5'GAAGCACACAGGAAAAGGAG3'), 0.5 mM lower primer (5'GACAAAGGTGACCCCAAG3') and 1x SYBR Green enzyme mix (Roche Diagnostic). GAPDH mRNA quantification included 100 ng cDNA, 3 mM $MgCl_2$, 0.5 mM upper primer (5'CCCATGTTCGTCATGGGTGT3'), 0.5 mM lower primer (5'TGGTCATGAGTCCTTCC ACGATA3') and 1x SYBR Green enzyme mix (Roche Diagnostic). PCR reactions were performed under the following conditions: ALXR: preheating of the mixture for 10 min at 95°C, followed by 45 cycles of denaturation for 5 sec at 95°C, annealing for 10 sec at 68°C and extension for 7 sec at 72°C. GAPDH: preheating of the mixture for 10 min at 95°C, followed by 45 cycles of denaturation for 5 sec at 95°C, annealing for 10 sec at 59°C and extension for 6 sec at 72°C. The primers were designed over exon/intron boundaries and amplified PCR products were checked for size on agarose gels and sequenced.

Generation of promoter constructs

Promoter constructs were amplified by PCR with XhoI upper primers and HindIII lower primers (supplementary table S1). Constructs were subcloned into the empty pGL3basic vector that contains the firefly luciferase gene (Promega, Rotkreuz, Switzerland) using the XhoI and HindIII restriction enzymes. Point mutations in transcription factor binding sites were introduced into P1-414 and P1-150 using the QuickChange® II XL site directed mutagenesis kit according to the manufacturer's instructions (Stratagene, Basel, Switzerland). The primers (Microsynth, Balgach, Switzerland) used to mutate P1-414 and P2-150, are listed in the supplementary table S2. All constructs were sequenced.

Transcription assays

For each experiment, $6X10^6$ THP-1 cells, 10 µg of construct and 0.25 µg of the internal control pHRL-SV40 (Promega) were used. Electroporation was done under the following conditions: 200 V, 950 µF capacitance and ∞ resistance. Following electroporation, cells were seeded in RPMI-1640 with 10 % FCS and 20 mM glutamine. Three hours later, cells were differentiated into macrophages with PMA for 48 hrs. For basal transcription assays, cells were washed with PBS and harvested. For the IFNγ-induced transcription assay, cells were stimulated another 24 hrs with 50 ng/ml of IFNγ in RPMI-1640 with 0.5 % FCS, washed with PBS and harvested. Harvested cells were lysed in 250 µl 1x passive lysis buffer and 50 µl were used for dual-luciferase reporter assays (Promega).

Preparation of nuclear extracts

Nuclear extracts were prepared one ice from THP-1 macrophages as described in (38). Briefly, cells were resuspended in 500 µl of Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0,1 mM EDTA. 0,1 mM EGTA, 1 mM DTT, 1 mM PMSF and protease inhibitors (Complete, Roche Diagnostics) and were incubated on a rotating wheel for 30 min at 4°C followed by the addition of 30 µl of 10% NP-40. After vortexing, the nuclei were pelleted by centrifugation for 5 min at 13000rpm. Nuclei were then resuspended in 60 µl of Buffer C (20mM HEPES pH 7.9, 25 % glycerol, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and protease inhibitors) and lysed by shaking for 15min. After centrifugation for 5 min at 13000 rpm, nuclear proteins in the supernatant were stored at -80°C until use. The protein concentration was measured with the M-TP-Reagent (Beckman Coulter, Nyon, Switzerland).

Electromobility shift assay (EMSA)

2 pmol of double stranded DNA probes corresponding to a potential transcription factor binding site were end-labelled with ^{32}P-γ-ATP (Hartmann Analytic, Germany) by T4 polynucleotide kinase (Fermentas, Le-Mont-sur-Lausanne, Switzerland). Sequences of the EMSA probes are listed in supplementary table S3. 10 µg of THP-1 macrophage nuclear extracts and 2 µg of freshly boiled polydI/dC were added to the binding buffer (24 mM HEPES pH 7.9. 24 % glycerol, 10 mM NaCl, 100 mM KCl, 10mM $MgCl_2$ and 1.2 mM DTT) in a 20 µl binding reaction. In the competition experiment unlabelled competitor was added in a 50X excess to the reaction. The labelled probes were added and the reaction was incubated for 20 min at room temperature. Samples were analyzed on a 4 % non-denaturing polyacrylamide gel (0.5x

TBE; 4 % acrylamide /bisacrylamide 29:1; 0.075 % APS, 2.5 % glycerol and TEMED). Gels were run at 150 V for 5 hrs, dried and analyzed with the scanner FLA-7000 and the software Image Gauge (Fujifilm, Basel, Switzerland). For the OCT1 probes, reactions were optimized with the promega binding buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 5 mM $MgCl_2$ and 20 % of glycerol). Binding reactions for IRSE used 0.75 µg of freshly boiled polydI/dC in binding buffer (20 mM HEPES pH 7.6; 1 mM EDTA, 1 mM DTT and 100 mM KCl). Following addition of the probes, the mixture was incubated for 20 min at 25°C as described in (39). For the supershift experiments 4 µg of SP1 antibody (sc-59), 4 µg or 10 µg of OCT1 (sc-25399) and 10 µg of IRF1 (sc-497), ICSBP (sc-13043) or IRF4 (sc-6059) were added 1hour prior to the labelled probes at 4°C. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Heidelberg, Germany)

Statistical analysis

Statistical analysis was performed with StatView version 5.0.1 (SAS Institute Inc., Cary, NC). The level of ALXR mRNA and the activities of the different ALXR promoters in the luciferase assays were compared using a two-sided t-test. A two-sided *p* value of <0.05 was considered significant.

Results

The *ALXR* gene has two promoters leading to two distinct transcription start sites

To define the transcriptional start site of the *ALXR* gene, 5' rapid amplification of cDNA ends was used in primary macrophages. The amplification produced two distinct bands of 320 and 600 bps (fig.1A, lane 2), which represent two mRNAs containing both the coding exon and the first exon of the *ALXR* gene but with one having a longer version of the first exon. These results suggest that the *ALXR* has two transcription start sites (fig.1B) and that *ALXR* transcription may be regulated by two promoters. To characterize the transcriptional activity of these two potential promoter regions, different sized fragments of the two regions were cloned upstream of a firefly luciferase reporter gene. As shown in fig.1C, all constructs containing promoter region P1 directed expression of the luciferase, and the shortest construct comprising 414 bps led to a 4-fold (4.5 ±1.5) increase in transcription compared to the empty vector. Similarly all constructs containing sequences of promoter region P2 directed expression of the luciferase with a 4-fold (3.8 ±0.3) increase in transcription of the shortest 150 bps promoter fragment. These results indicate that one core promoter each is located upstream of both transcription start sites.

Both *ALXR* promoters are recognized by basal transcription factors

To characterize basal transcription by the two TATA-less promoters P1 and P2 of the *ALXR* gene, we analyzed these regions for transcription factor binding sites using the bioinformatic tools MatInspector (www.genomatix.de) and Alibaba (www.gene-regulation.com). We identified eight putative transcription factor binding sites in promoter P1 implicated in the formation of the transcriptional pre-initiation complex of TATA-less promoters, namely one PU1, SP1, NFY, GATA, SP2 and three OCT1 binding sites. To identify the role of these transcription factor binding sites on basal transcription, we performed site-directed mutagenesis of their core binding sites (supplementary table S4). These experiments revealed that an OCT1 binding site supports the basal transcription activity of promoter P1. A single C to A substitution in the OCT1 core binding site at position -103 resulted in an almost 50 % reduction of luciferase expression (47 %±29 %) compared to the wild-type promoter P1-414 (fig.2A).

To verify that OCT1 binds to this sequence in promoter P1, we performed EMSA analysis with THP-1 nuclear extracts. Specific binding of the nuclear extracts to this OCT1 oligonucleotide was shown (fig.2B lane1) which could be competed in the presence of unlabelled wildtype but not mutant oligonucleotide (fig.2B lane 2, OCT1 and lane 3, mOCT1). The presence of the A to C mutation at position -103 also dramatically reduced binding of nuclear proteins (fig.2B lane 5). To show that OCT1 is part of the protein complex binding to the OCT1 oligonucleotide, nuclear extracts were co-incubated with an OCT1 antibody. The observed supershift demonstrates that OCT1 is binding to the OCT1 transcription factor binding site in promoter P1 (fig.2B lane 4). This finding is further supported by the similar supershift observed with the designed OCT1 oligonucleotide used as a positive control (fig.2B lane 6).

We also identified two putative basal transcription factor binding sites in promoter P2, namely a NFY and SP1 binding site. Mutations in the core binding site of both NFY and SP1 (supplementary table S4) resulted in a 83 % (±3 %) and 91 % (±3 %) reduction of promoter

activity compared to the wild-type promoter P2-150, respectively (fig.2C). To confirm the binding of NFY and SP1 to promoter P2, we again performed EMSA analysis with THP-1 nuclear extracts. Direct binding of nuclear proteins to the SP1 and NFY oligonucleotides was confirmed (fig.2D lane 1 and lane 6) and a decrease in binding was observed for both transcription factor binding sites when the core binding site was mutated (fig.2D lane 9 and 10). Binding to the SP1 oligonucleotide could be competed with unlabelled SP1 oligonucleotide (fig.2D lane 2, SP1) but not with mutant unlabelled SP1 oligonucleotide (fig.2D lane 4, mSP1). However, the NFY oligonucleotide also competed with the SP1 oligonucleotide and vice versa (fig.2D lane 3, NFY and 7, SP1), suggesting that these overlapping transcription factor binding sites are recognized by the same protein complex. Co-incubation of the nuclear extracts with a SP1 antibody supported this finding, showing the identical supershift for both SP1 and NFY oligonucleotides (fig.2D lane 5 and 8 respectively), while no supershift was observed with an antibody against NFY (data not shown). Taken together, these results indicate that SP1 is part of the complex binding the core promoter region P2 to activate basal transcription.

ALXR mRNAs is downregulated during monocyte differentiation and upregulated in macrophages by IFNγ

In an effort to understand the role of ALXR in macrophages during inflammation we investigated the level of ALXR mRNA during the differentiation of primary monocytes to macrophages. Our results show that monocytes but not 7 days old macrophages express ALXR mRNA (fig.3A). These results indicate that macrophages need external stimuli to produce ALXR. Such an upregulation of the ALXR mRNAs was shown for different types of cytokines, however, whether this is true in macrophages has not been investigated. To answer this question, primary macrophages were stimulated with several cytokines known to be secreted during inflammation (fig.3B). 50 ng/ml of the pro-inflammatory cytokine IFNγ resulted in an 80-fold increase in the expression of ALXR mRNA compared to the non-stimulated cells, whereas stimulation with the other pro- and anti-inflammatory cytokines showed no alteration of ALXR mRNA expression. Thus IFNγ-stimulation led to the rescue of mRNA production lost during the differentiation to macrophages (data not shown). In addition, increasing concentrations of INFγ showed that the effect on ALXR mRNA was dose-dependent (fig.3C). Taken together these results indicate that ALXR is tightly regulated and that IFNγ recruits the transcriptional machinery to increase promoter activity and thus ALXR mRNA expression.

INFγ increases transcriptional activity of the first promoter via an IRSE element

To locate the IFNγ response element in the *ALXR* promoter, THP-1 macrophages were subjected to 24 hrs stimulation with IFNγ after electroporation of the different constructs of promoters P1 and P2. As seen in fig.4A IFNγ-stimulation resulted in a 1.5-fold (±0.6) increase in luciferase expression for the first promoter P1 compared to non-stimulated cells, whereas IFNγ had no stimulatory effect on the second promoter P2. Theses results suggest that P1 contains the transcriptional elements which promote the IFNγ dependent stimulation of the *ALXR* gene. MatInspector located two putative binding sites for IRF4 and IRF7 within this region. These transcription factors have already been shown to induce transcription upon stimulation with interferons (40,41). To investigate whether one or both of these transcription factor binding sites are necessary for the IFNγ response of the *ALXR* gene, their core binding sites were mutated on the shortest P1-414 luciferase-construct (supplementary table S4). Site-directed mutagenesis of the core binding site for IRF4 resulted in a 48 % decrease in basal transcriptional activity compared to the wildtype construct, while site-directed mutagenesis of IRF7 did not alter the promoter activity (fig.4B). In addition, when cells were stimulated with IFNγ, the construct containing the mutant IRF4 binding site showed decreased transcription even compared to non-stimulated cells (p=0.038) (fig.4C). All together these results indicate that the transcription factor binding site for IRF4 is important for basal and IFNγ-induced transcription of *ALXR*. EMSA confirmed binding of proteins from nuclear extracts from non-stimulated (fig.4D, lane1) and even more from IFNγ-stimulated macrophages (fig.4D, lane2) to the IRF4 oligonucleotide. Interestingly, stimulation of macrophages with IFNγ changed the size of DNA-protein complexes indicating differential regulation for basal and induced transcription. This binding could be competed with unlabelled IRF4 oligonucleotide (fig.4D, lane 3, IRF4) but less so with a mutant version (fig.4D, lane 4, mIRF4), supporting a role for this transcription factor binding site in IFNγ

stimulation. However, incubation of the IRF4 oligonucleotide with nuclear extracts in the presence of an IRF4 antibody did not result in a supershift of the protein-DNA complex (fig.4D, lane 7), arguing against an involvement of IRF4 in IFNγ-stimulation of the *ALXR* gene. Since members of the IRF family of transcription factors bind to interferon-stimulated response elements (IRSE) (42) and the IRF4 site also matches this IRSE consensus sequence, we probed the protein-DNA complexes with several antibodies against transcription factors of the IRF family. As can be seen in figure 4D, only incubation with the IRF1 antibody resulted in a supershift of the protein-DNA complexes (lane 10). All together, these results indicate that IFNγ stimulates the transcription of the *ALXR* gene via IRF1 binding to the ISRE element located in the first promoter P1.

Discussion

We identified and characterized two promoter regions in the *ALXR* gene separated by 224 bps which drive the ALXR mRNA expression in monocytes and macrophages. We also show that transcription of the *ALXR* gene from these promoters decreases during differentiation of primary human monocytes to macrophages and that the low transcription in macrophages can be rescued by IFNγ-stimulation. On the molecular level, we have identified that the transcription factors OCT1 and SP1 are involved in the initiation of transcription in the TATA-less promoter 1 and 2 of the *ALXR* gene, respectively. We have further located the IFNγ response element to promoter 1 and have shown that for IFNγ-stimulation of ALXR mRNA transcription the transcription factor IRF1 binds an IRES transcription factor binding site in promoter 1.

Two distinct promoters are located in the *ALXR* gene which result in alternative 5'UTRs of the ALXR mRNA in macrophages. Both promoters lack a TATA box at position -25 but have basal transcription factor binding sites present, which were previously shown to initiate transcription of TATA-less promoters. The promoter P1 contains an OCT1 binding site which has previously been shown to play a critical role in the pre-initiation complex recruitment of promoters that lack TATA boxes. For instance, OCT1 was able to replace TBP via its interaction with the general transcription factor TFIIB for the transcription of the lipoprotein lipase gene (43). Similarly, the promoter P2 contains a basal transcription factor binding site for SP1, which is known to bind CpG islands and also to drive gene transcription of TATA-less promoters (44). Both of these promoters seem to initiate basal transcription of the *ALXR* gene in monocytes and less so in macrophages but only the first promoter P1 has the necessary transcription factor binding sites for IFNγ-stimulation.

This first promoter P1 is responsive to IFNγ-stimulation and contains an IRSE element recognized by the transcription factor IRF1. The signalling pathways of IFNγ-stimulation involves either the activation of the JAK/STAT pathway or an increase in transcription of Interferon Regulatory Factors (IRF) (45), which both leads to enhanced transcription of downstream genes. Here we show that IRF1 supports the increased *ALXR* expression through binding to an IRSE binding site (46) in the first promoter of the *ALXR* gene. IRF1 is one of the highest induced members of the IRF family and was shown to be highly upregulated by IFNγ in THP-1 monocytes (47). This resulted in a strong increase in IRF1 binding to the IRSE element in the β2-microglobulin promoter (47) similar to the binding shown in our EMSA experiment with the IRSE element in the first promoter P1 of the *ALXR* gene.

Our finding that the ALXR is expressed to high levels in monocytes supports the role of the ALXR in chemotaxis, while the observed downregulation of the mRNA during differentiation limits its role in macrophages. The ALXR plays a role in chemotaxis of monocytes during inflammation, which results from a gradient of pro-inflammatory or anti-inflammatory ligands. Depending on the ligand and the environment of the invaded tissue, the monocyte may then differentiate into either a pro-inflammatory M1 or a non-phlogistic M2 macrophage. For example, SAA accelerates inflammation through the ALXR and has been shown to stimulate monocytes to produce the pro-inflammatory chemokine CCL2 (6) and TNFα (48). In contrast, treatment of monocytes with the ALXR ligand LXA_4 was shown to recruit monocytes to the site of inflammation without leading to cytotoxicity and superoxide generation (18,49). These monocytes differentiated into pro-resolving M2 macrophages, which take up apoptotic cells from the inflamed tissue and support tissue homeostasis. Therefore, high expression of ALXR in monocytes may reflect its role in signalling chemotactic stimuli but also in signalling pro- or anti-inflammatory effects depending on the environment.

During differentiation of monocytes to macrophages expression of the *ALXR* gene decreases and only stimulation of macrophages

with IFNγ rescues *ALXR* transcription, while other pro- and anti-inflammatory cytokines had no effect on ALXR mRNA levels. These data suggest that only the subset of M1 macrophages stimulated with IFNγ will respond to ALXR ligands, while other classes of resident macrophages will not. IFNγ is an endogenous mediator of immunity and inflammation and plays a complex and central role in the resistance of the mammalian host to pathogens. IFNγ stimulation of monocytes and macrophages leads to the development of classical activated M1 macrophages, which display enhanced endocytic functions and have the ability to kill intracellular pathogens (50,51). Thus it is likely that other pro-inflammatory mediators like the ALXR ligand SAA are present in concert with IFNγ, suggesting that the induced expression of ALXR in these M1 macrophages will accelerate inflammation. Nevertheless, upregulation of ALXR in macrophages by IFNγ may also lead to the resolution of inflammation and tissue repair, when the anti-inflammatory ligands annexin1 or LXA4 rise in concentration during the resolution phase of inflammation.

While cell surface expression of the ALXR on monocytes has recently been demonstrated (52), data on cell surface expression of ALXR on macrophages are lacking. We were able to detect ALXR expression on the cell surface in monocytes by FACS analysis and could confirm its absence on macrophages. However, we could not detect ALXR cell surface expression in macrophages stimulated with IFNγ. This may be due to technical problems of detaching macrophages from cell culture dishes, although we could detect the presence of other cell surface receptors on macrophages (data not shown). Western blot analysis was also not successful because none of several commercial antibodies was specific for ALXR. Another explanation for this discrepancy of mRNA and cell surface expression of the protein may be that post-transcriptional mechanisms regulate ALXR abundance on the cell surface. Our data indicate that IFNγ-stimulated transcription of the *ALXR* gene leads to the mRNA containing the longer 5'UTR because only the first promoter contains the IRES element and is responsive to IFNγ-stimulation. The 5'UTRs are known to contain regulatory motifs (53,54) that have an effect on translational efficiency, like target sequences for MicroRNAs (55), internal ribosome entry sites (56), hairpin secondary structures (57), upstream translation initiation codons (uAUG) and upstream open reading frames (uORF). In this line, the longer 5'UTR of the ALXR mRNA derived from promoter P1 contains an uORF which may lead to a poor translation of this ALXR mRNA (58). This is supported by recent studies which revealed that the presence of an uORFs in human genes results in a 30-80% reduction in protein expression or even in a full block of translation (53,59-61). Hence, IFNγ stimulation of macrophages may result in ALXR mRNA containing the uORF which may not be efficiently translated and therefore not support membrane expression of the receptor.

In summary, we have characterized the regulatory sequences and some components of the transcriptional machinery that drives the expression of the *ALXR* gene in monocytes and macrophages. Our data also suggest that the ALXR mRNA is expressed in monocytes but only in a limited set of macrophages stimulated with IFNγ. This supports the current model that the ALXR is a chemotactic receptor in monocytes, however, limits its role in macrophages to the IFNγ stimulated pro-inflammatory macrophages.

Aknowlegdment

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Legends

Figure 1: **Location of the *ALXR* promoters in macrophages**. A: PCR products are visualized on a 2 % agarose gel after nested 5'RACE. Lane 1: Gene specific nested amplification (forward nested gene specific primer and reverse gene specific inner primer). Lane 2: Race specific nested amplification (forward 5'RACE adapter nested inner primer and reverse gene specific inner primer). B: Genomic sequence including the ALXR promoters with the two transcription start sites indicated as +1. Transcription factor binding sites identified for AXLR transcription are underlined. C: Promoter constructs were cloned into a pGL3 basic luciferase vector and are referenced as numbers in base pairs relative to +1 for the first promoter P1 and for the second promoter P2. Human THP-1 monocytes were transfected with the constructs, differentiated into macrophages and luciferase activities were measured 48 hrs after transfection. Firefly luciferase activities were normalized to Renilla luciferase activities and all experiments were done in triplicates. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over the empty vector pGL3b and the error bars display standard deviation (± SD). *p<0.05; **p<0.01.

Figure 2: **Identification of basal transcription factor binding sites in the *ALXR* promoter region.** Putative basal transcription factor binding sites for P1-414 and P2-150 were identified with the softwares Genomatix and Alibaba. A. and C: Individual transcription factor binding sites were mutated in P1-414 (A) and P2-150 (C). For mutations introduced see supplementary table S4. Human THP-1 monocytes were transfected with the constructs, differentiated into macrophages and luciferase activities were measured 48 hrs after transfection. Firefly luciferase activities were normalized to Renilla luciferase activities and all experiments were done in triplicate. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over P1-414 and P2-150, respectively. B. Electrophoretic mobility shift assays for the potential OCT1 binding site with nuclear extracts from THP-1 macrophages. The DNA-protein complex with OCT1 oligonucleotides was analyzed by gel electrophoresis and visualized by autoradiography (lane 1). For competition assays, the nuclear extracts were preincubated with excess of unlabeled OCT1 or mutant OCT1 oligonucleotides (OCT1: lane 2; mOCT1: lane 3). For supershift analysis, the nuclear extracts were preincubated with an OCT1 antibody (lane 4). The nuclear proteins were incubated with the mutant OCT1 oligonucleotide to test direct binding (lane 5). A control of supershift was done with OCT1 antibody and a designed OCT1 oligonucleotide (lane 6). D. Electrophoretic mobility shift assay for SP1 and NFY. DNA-protein complexes with SP1 and NFY oligonucleotides were visualized by autoradiography (lane1 and lane 6, respectively). Competition assays were performed with three competitors for SP1: SP1 (lane 2), NFY (lane 3) and mutant SP1 (mSP1: lane 4). One competitor was used for NFY: SP1 (lane 7). SP1 antibody was used for supershift assays with the SP1 and NFY oligonucleotides (lane 5 and lane 8). The nuclear extracts were incubated with the mutant SP1 (mSP1: lane 9) and the mutant NFY oligonucleotides (mNFY: lane 10) to see abrogated direct binding. Sequences of the different oligonucleotides can be found in the supplementary table S3. Error bars display standard deviation (± SD) *p<0.05; **p<0.01. Ab: supershift with antibody.

Figure 3: **Regulation of ALXR mRNA expression.** ALXR mRNA was determined by real-time RT-PCR in primary monocytes and macrophages. A: Monocytes extracted from buffy coat (day 0) were allowed to differentiate into macrophages for 1, 3, 6, and 7days. ALXR mRNA values were normalized to GAPDH mRNA levels and results represent fold induction compared to monocyte expression levels at day 0. B: Total RNA was extracted from 7 days old macrophages stimulated for 24 hrs with IL-13, IL-1β, TNFα, TGFβ, and IFNγ. Values represent fold induction compared to the non-stimulated cells. C: Ratio of ALXR/GAPDH expression levels of cells stimulated with different concentrations of IFNγ (-: no stimulation; 10 ng/ml; 25 ng/ml; 50 ng/ml). Error bars display standard deviation (± SD); n=3; *p<0.05; **p<0.01.

Figure 4: **Location of the IFNγ response element.** A: THP-1 monocytes were transfected with the different constructs of promoter P1 and P2 and differentiated into macrophages for 2 days. Cells were further stimulated with IFNγ for 24 hrs and luciferase activities were measured and normalised to the activity of the Renilla luciferase. Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction of the respective construct compared to non-stimulated cells. B and C: Site-directed mutagenesis of the putative interferon response elements IRF4 and IRF7 in construct P1-414 (for mutations see supplementary table S4). Luciferase activity was

measured 48 hrs after transfection (B) and following 24 hrs stimulation with IFNγ (C). Bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction over the P1-414 construct (B) or over the respective construct in non-stimulated cells. D: EMSA for IRF4 with the nuclear extracts from THP-1 macrophages (lane 1) and THP-1 macrophages stimulated for 24 hrs with IFNγ (lane 2). For competition assays, the stimulated nuclear extracts were preincubated with excess of unlabeled IRF4 (lane 3) or mutant IRF4 oligonucleotides (mIRF4: lane 4). As the binding site overlaps with a putative OCT1 transcription factor binding site, nuclear extracts from non-stimulated cells were preincubated with an excess of unlabelled designed OCT1 oligonucleotide (lane 5). The nuclear extracts from stimulated macrophages were incubated with the mutant IRF4 oligonucleotide to test direct binding (lane 6). For supershift assays IRF4, ISBCP, OCT1 and IRF1 antibodies were preincubated with the IRF4 oligonucleotide (lane 7, 8, 9 and 10). Sequences of oligonucleotides can be found in the supplementary table S3. Error bars display standard deviation (± SD). *$p<0.05$; **$p<0.01$. Ab: supershift with antibody.

Figure 1:

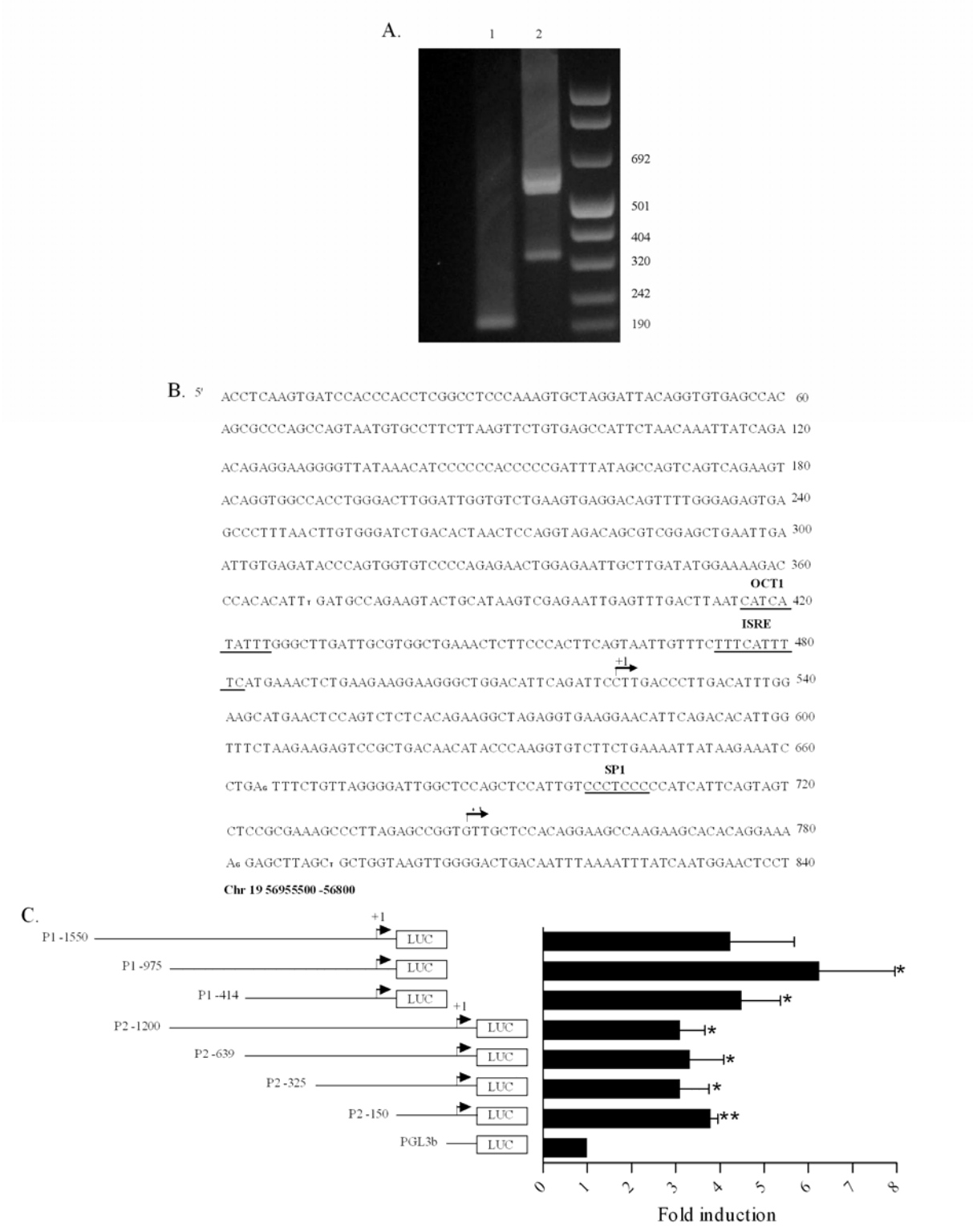
A.
1
2
692
501
404
320
242
190
B. 5'
ACCTCAAGTGATCCACCCACCTCGGCCTCCCAAAGTGCTAGGATTACAGGTGTGAGCCAC 60
AGCGCCCAGCCAGTAATGTGCCTTCTTAAGTTCTGTGAGCCATTCTAACAAATTATCAGA 120
ACAGAGGAAGGGGTTATAAACATCCCCCCACCCCCGATTTATAGCCAGTCAGTCAGAAGT 180
ACAGGTGGCCACCTGGGACTTGGATTGGTGTCTGAAGTGAGGACAGTTTTGGGAGAGTGA 240
GCCCTTTAACTTGTGGGATCTGACACTAACTCCAGGTAGACAGCGTCGGAGCTGAATTGA 300
ATTGTGAGATACCCAGTGGTGTCCCCAGAGAACTGGAGAATTGCTTGATATGGAAAAGAC 360
OCT1
CCACACATT GATGCCAGAAGTACTGCATAAGTCGAGAATTGAGTTTGACTTAATCATCA 420
ISRE
TATTTGGGCTTGATTGCGTGGCTGAAACTCTTCCCACTTCAGTAATTGTTTCTTTCATTT 480
+1
TCATGAAACTCTGAAGAAGGAAGGGCTGGACATTCAGATTCCTTGACCCTTGACATTTGG 540
AAGCATGAACTCCAGTCTCTCACAGAAGGCTAGAGGTGAAGGAACATTCAGACACATTGG 600
TTTCTAAGAAGAGTCCGCTGACAACATACCCAAGGTGTCTTCTGAAAATTATAAGAAATC 660
SP1
CTGA TTTCTGTTAGGGGATTGGCTCCAGCTCCATTGTCCCTCCCCCATCATTCAGTAGT 720
+1
CTCCGCGAAAGCCCTTAGAGCCGGTGTTGCTCCACAGGAAGCCAAGAAGCACACAGGAAA 780
A GAGCTTAGC GCTGGTAAGTTGGGGACTGACAATTTAAAATTTATCAATGGAACTCCT 840
Chr 19 56955500 -56800
C.
+1
P1 -1550
P1 -975
P1 -414
+1
P2 -1200
P2 -639
P2 -325
P2 -150
PGL3b
LUC
*
**
0
1
2
3
4
5
6
7
8
Fold induction

Figure2:

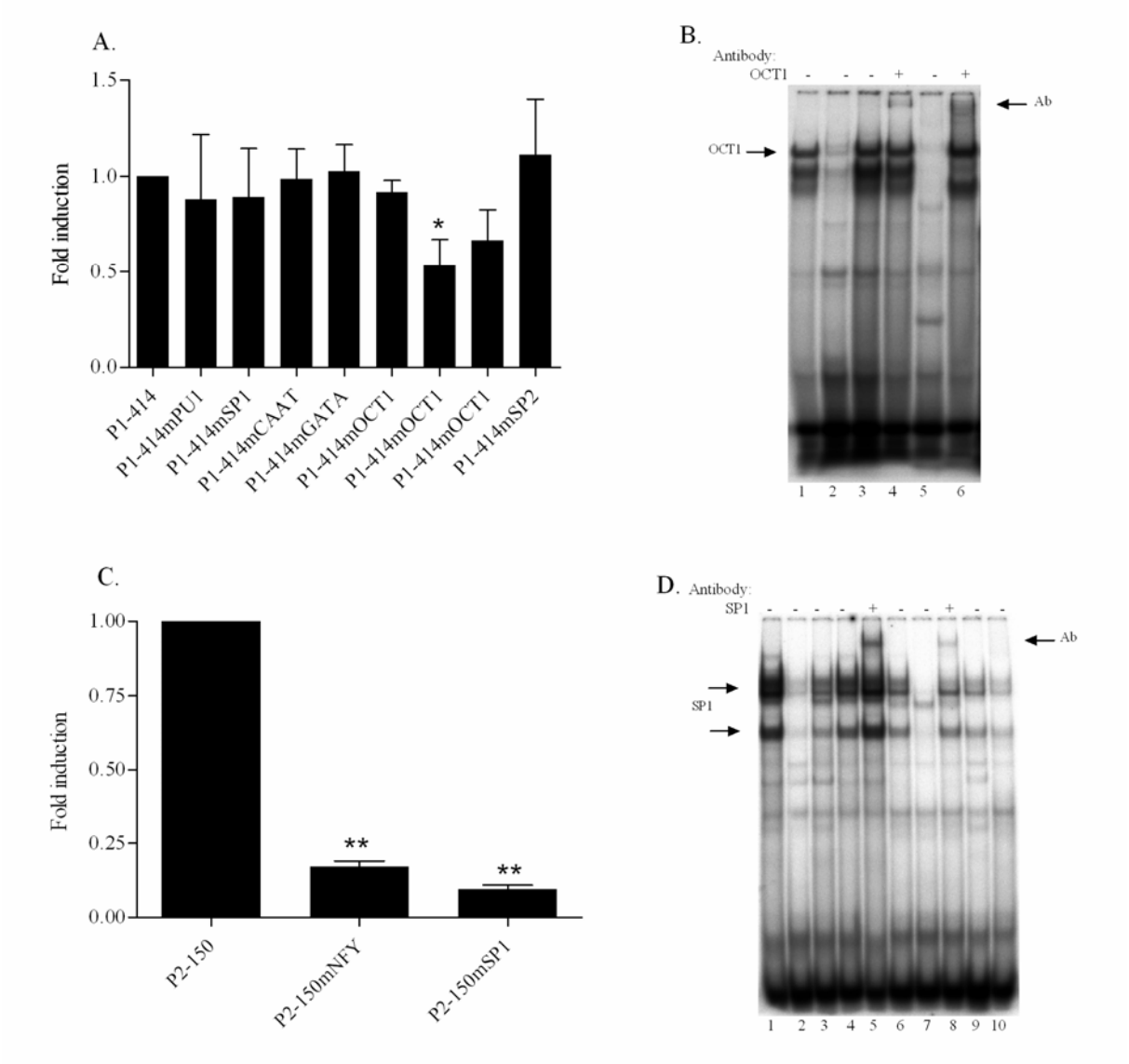
A.
Fold induction
1.5
1.0
0.5
0.0
*
P1-414
P1-414mPU1
P1-414mSP1
P1-414mCAAT
P1-414mGATA
P1-414mOCT1
P1-414mOCT1
P1-414mOCT1
P1-414mSP2
B.
Antibody:
OCT1 - - - + - +
Ab
OCT1
1 2 3 4 5 6
C.
Fold induction
1.00
0.75
0.50
0.25
0.00
**
**
P2-150
P2-150mNFY
P2-150mSP1
D.
Antibody:
SP1 - - - - + - - + - -
Ab
SP1
1 2 3 4 5 6 7 8 9 10

Figure 3:

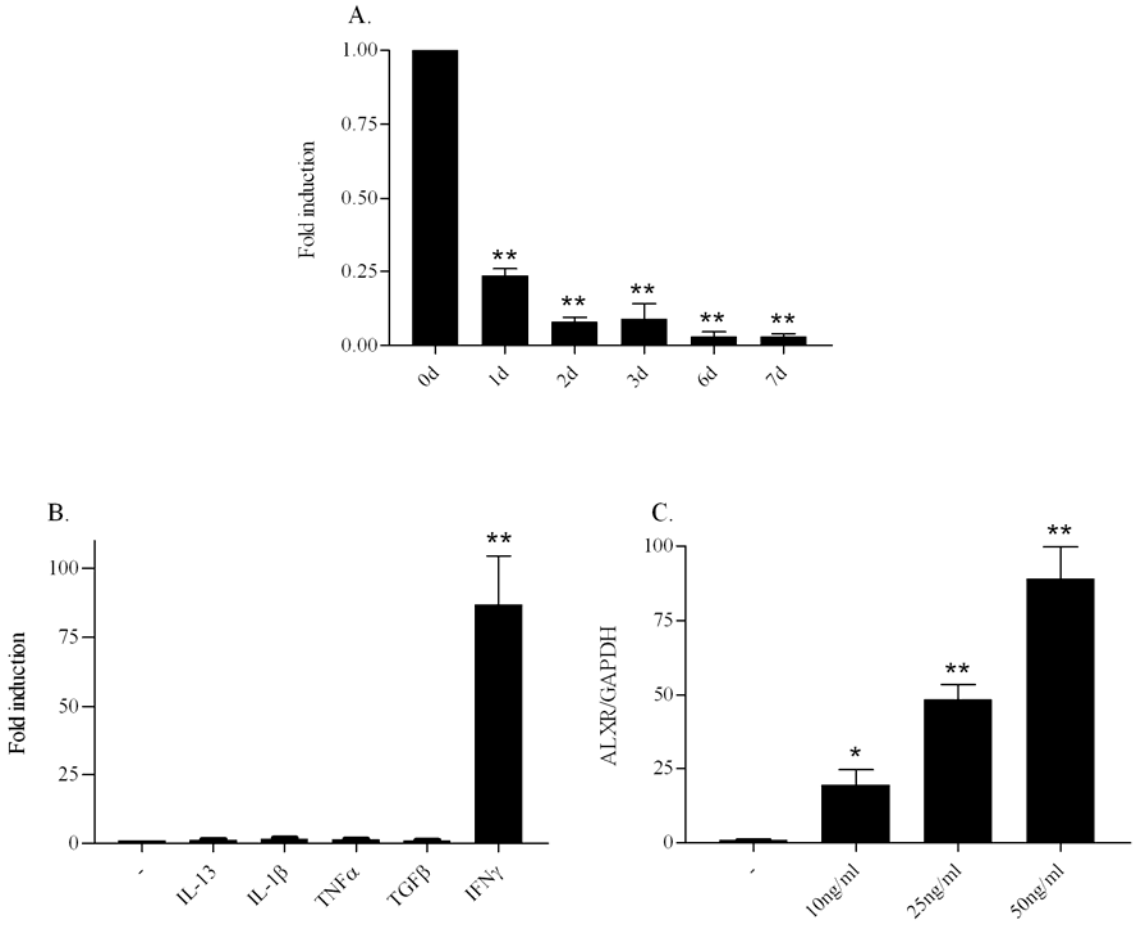
A.
Fold induction
1.00
0.75
0.50
0.25
0.00
**
0d
1d
2d
3d
6d
7d
B.
Fold induction
100
75
50
25
0
**
-
IL-13
IL-1β
TNFα
TGFβ
IFNγ
C.
ALXR/GAPDH
100
75
50
25
0
*
**
**
-
10ng/ml
25ng/ml
50ng/ml

Figure 4:

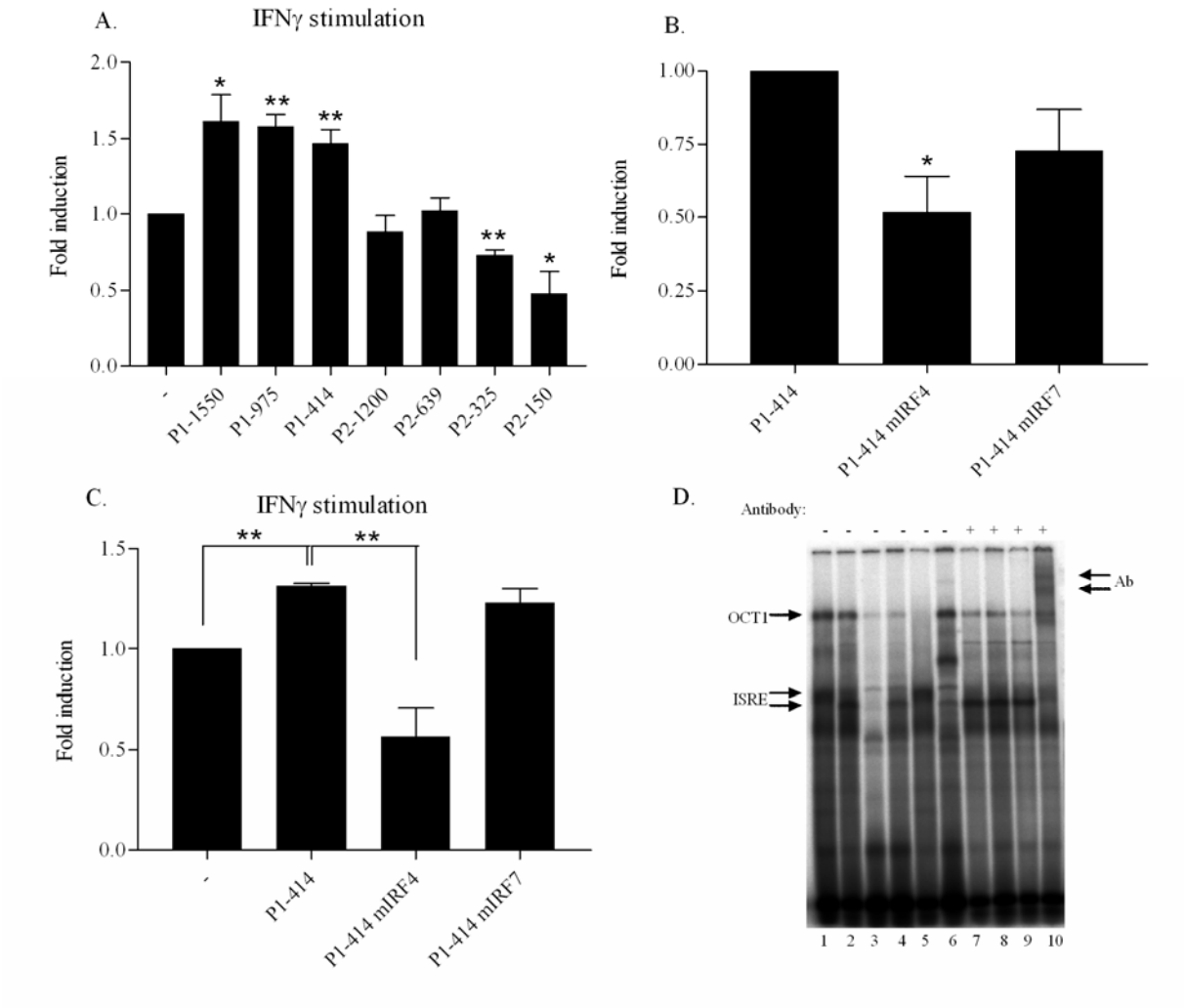
A.
IFNγ stimulation
Fold induction
2.0
1.5
1.0
0.5
0.0
*
**
**
**
*
-
P1-1550
P1-975
P1-414
P2-1200
P2-639
P2-325
P2-150
B.
Fold induction
1.00
0.75
0.50
0.25
0.00
*
P1-414
P1-414 mIRF4
P1-414 mIRF7
C.
IFNγ stimulation
Fold induction
1.5
1.0
0.5
0.0
**
**
-
P1-414
P1-414 mIRF4
P1-414 mIRF7
D.
Antibody:
- - - - - - + + + +
Ab
OCT1
ISRE
1 2 3 4 5 6 7 8 9 10

Table S1: **Primers sequences used for plasmid constructs.**

Promoter constructs	Primers (5'-3')	
P1-1550	upstream primer:	CACTCATACTCGAGATGGGAATTGAACAATGAGGAAAC
	downstream primer:	ACACCTTGAAGCTTGGTATGTTGTCAGCGGACTCTCTTCTTAG
P1-975	upstream primer:	ATCACTACTCGAGCGTGCCCCACCCTATTGTAACT
	downstream primer:	ACACCTTGAAGCTTGGTATGTTGTCAGCGGACTCTCTTCTTAG
P1-414	upstream primer:	GCCATTCTCTCGAGAACAAATTATCAGAACAGAGGAAGGGG
	downstream primer:	ACACCTTGAAGCTTGGTATGTTGTCAGCGGACTCTCTTCTTAG
P2-1200	upstream primer:	ATCACTACTCGAGCGTGCCCCACCCTATTGTAACT
	downstream primer:	GTGTGCTAAGCTTTCTTGGCTTCCTGTGGAGCAA
P2-639	upstream primer:	GCCATTCTCTCGAGAACAAATTATCAGAACAGAGGAAGGGG
	downstream primer:	GTGTGCTAAGCTTTCTTGGCTTCCTGTGGAGCAA
P2-325	upstream primer:	ATCATCACTCGAGTATTTGGGCTTGATTGCGTGGCT
	downstream primer:	GTGTGCTAAGCTTTCTTGGCTTCCTGTGGAGCAA
P2-150	upstream primer:	CAGACACCTCGAGATTGGTTTCTAAGAAGAGTCCG
	downstream primer:	GTGTGCTAAGCTTTCTTGGCTTCCTGTGGAGCAA

Table S2: **Sequences of primers used for site-directed mutagenesis**.

Transcription factor	Position (a)	Primers (5'-3')
PU1	P1 -412/-370	upstream primer: CAAATTATCAGAACAGAAGAAGGGGTTATAAACATCCCCCCA
		downstream primer: TGGGGGGATGTTTATAACCCCTTCTTCTGTTCTGATAATTTG
SP1	P1 -395/-353	upstream primer: GGAAGGGGTTATAAACATCCCCCCACTCCCGATTTATAGCCA
		downstream primer: TGGCTATAAATCGGGAGTGGGGGGATGTTTATAACCCCTTCC
CAAT	P1 -377/-353	upstream primer: CCCCCCACCCCCGATTTATAGGCAGTCAGTCAGAAGTACAGG
		downstream primer: CCTGTACTTCTGACTGACTGCCTATAAATCGGGGGTGGGGGG
GATA	P1 -189/-147	upstream primer: ACTGGAGAATTGCTTCATATGGAAAAGACCCACACATTTGAT
		downstream primer: ATCAAATGTGTGGGTCTTTTCCATATGAAGCAATTCTCCAGT
OCT1	P1-189/-147	upstream primer: ACTGGAGAATTGCTTGATACGGAAAAGACCCACACATTTGAT
		downstream primer: ATCAAATGTGTGGGTCTTTTCCGTATCAAGCAATTCTCCAGT
OCT1	P1 -125/-83	upstream primer: AGAATTGAGTTTGACTTAATCCTCATATTTGGGCTTGATTGC
		downstream primer: GCAATCAAGCCCAAATATGAGGATTAAGTCAAACTCAATTCT
IRF4	P1 -61/-21	upstream primer: CAGTAATTGTTTCTATCATTTTCATGAAACTCTGAAGAAG
		downstream primer: CTTCTTCAGAGTTTCATGAAAATGATAGAAACAATTACTG
OCT1	P1-57/-15	upstream primer: AATTGTTTCTTTCATTTTCTTGAAACTCTGAAGAAGGAAGGG
		downstream primer: CCCTTCCTTCTTCAGAGTTTCAAGAAAATGAAAGAAACAATT
SP2	P1 -23/+17	upstream primer: AGGAAGGGCTGTACATTCAGATTCCTTGACCCTTGACATT
		downstream primer: AATGTCAAGGGTCAAGGAATCTGAATGTACAGCCCTTCCT
IRF7	P1 -24/+16	upstream primer: AAGGAAGGGCTGGACATTCAGATCCCTTGACCCTTGACAT
		downstream primer: ATGTCAAGGGTCAAGGGATCTGAATGTCCAGCCCTTCCTT
NFY	P2 -83/-41	upstream primer: GAGTTTCTGTTAGGGGATTGACTCCAGCTCCATTGTCCCTCC
		downstream primer: GGAGGGACAATGGAGCTGGAGTCAATCCCCTAACAGAAACTC
SP1	P2 -67/-22	upstream primer: ATTGGCTCCAGCTCCATTGTCCATACCCCATCATTCAGTAGT CTC
		downstream primer: GAGACTACTGAATGATGGGGTATGGACAATGGAGCTGGAGCCAAT

(a). Position relative to the transcriptional start site of the respective promoter P1 or P2

Table S3: **Oligonucleotides used for electromobility shift assay.**

Transcription factor	Position (a)	Primers (5'-3')
OCT1	P1 -125/-83	upstream primer: GTTTCTTTCATTTTCATGAAACTCTGAAGA downstream primer: TTCTTCAGAGTTTCATGAAAATGAAAGAAA
mOCT1	P1 -125/-83	upstream primer: GTTTCTTTCATTTTCTTGAAACTCTGAAGA downstream primer: TTCTTCAGAGTTTCAAGAAAATGAAAGAAA
IRF4	P1 -61/-21	upstream primer: CAGTAATTGTTTCTTTCATTTTCATGAAAC downstream primer: AGTTTCATGAAAATGAAAGAAACAATTACT
mIRF4	P1 -61/-21	upstream primer: CAGTAATTGTTTCTATCATTTTCATGAAAC downstream primer: AGTTTCATGAAAATGATAGAAACAATTACT
NFY	P2 -83/-41	upstream primer: TCTGTTAGGGGATTGGCTCCAGCTCCATTG downstream primer: ACAATGGAGCTGGAGCCAATCCCCTAACAG
mNFY	P2 -83/-41	upstream primer: TCTGTTAGGGGATTGACTCCAGCTCCATTG downstream primer: ACAATGGAGCTGGAGTCAATCCCCTAACAG
SP1	P2 -67/-22	upstream primer: CAGCTCCATTGTCCCTCCCCCATCATTCAG downstream primer: ACTGAATGATGGGGGAGGGACAATGGAGCT
mSP1	P2 -67/-22	upstream primer: CAGCTCCATTGTCCATACCCCATCATTCAG downstream primer: ACTGAATGATGGGGTATGGACAATGGAGCT
OCT1 promega	-	upstream primer: TGTCGAATGCAAATCACTAGAA downstream primer: ACAGCTTACGTTTAGTGATCTT

(a). Position relative to the transcriptional start site of the respective promoter P1 or P2

Table S4: **Putative transcription factor binding site of the *ALXR* promoter regions**.

Transcription factor	Position (a)	Transcription factor / Sequence (b)	
PU1	P1 -412/-370	PU1:	caaattatcagaacaga GGAAggggttataaacatcccccca
		m PU1:	caaattatcagaacaga AGAAggggttataaacatcccccca
SP1	P1 -395/-353	SP1:	ggaaggggttataaacatccc cccACCCccgatttatagcca
		m SP1:	ggaaggggttataaacatccc cccACTCccgatttatagcca
CAAT	P1 -377/-353	CAAT:	ccccccaccccccgatttatag CCAGtcagtcagaagtacagg
		m CAAT:	ccccccaccccccgatttatag GCAGtcagtcagaagtacagg
GATA	P1 -189/-147	GATA:	actggagaattgctt GATAtggaaaagacccacacatttgat
		m GATA:	actggagaattgctt CATAtggaaaagacccacacatttgat
OCT1	P1-189/-147	OCT1:	actggagaattgcttga TATGgaaaagacccacacatttgat
		m OCT1 :	actggagaattgcttga TACGgaaaagacccacacatttgat
OCT1	P1 -125/-83	OCT1:	agaattgagtttgacttaat CATCatatttgggcttgattgc
		m OCT1:	agaattgagtttgacttaat CCTCatatttgggcttgattgc
IRF4	P1 -61/-21	IRF4:	cagtaattgtttc TTTCattttcatgaaactctgaagaag
		m IRF4:	cagtaattgtttc TATCattttcatgaaactctgaagaag
OCT1	P1-57/-15	OCT1 :	aattgtttctt tcatttTCATgaaactctgaagaaggaaggg
		m OCT1:	aattgtttctt tcatttTCTTgaaactctgaagaaggaaggg
SP2	P1 -23/+17	SP2:	aggaa gggctGGACattcagattccttgacccttgacatt
		m SP2:	aggaa gggctGTACattcagattccttgacccttgacatt
IRF7	P1 -24/+16	IRF7:	aaggaagggctggaca ttcagATTCcttgacccttgacat
		m IRF7:	aaggaagggctggaca ttcagATCCcttgacccttgacat
NFY	P2 -83/-41	NFY:	gagtttctgttagggga TTGGctccagctccattgtccctcc
		m NFY:	gagtttctgttagggga TTGActccagctccattgtccctcc
SP1	P2 -67/-22	SP1:	attggctccagctccattgt cccTCCCccatcattcagtagtctc
		m SP1:	attggctccagctccattgt ccaTACCccatcattcagtagtctc

(a). Position relative to the transcriptional start site of the respective promoter P1 or P2
(b). Sequence used for site-directed mutagenesis; in red nucleotides important for transcription factor binding, in capital their core binding site and in blue nucleotide change used for site-directed mutagenesis.

2.2 **Paper**

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No association of two functional polymorphisms in human ALOX15 with myocardial infarction.

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Contribution:

1. Part in the conceptual design of the study
2. Part in the selection of the SNPs analyzed in ALOX15 to cover the most frequent haplotypes
3. Performed experiments for analysis of the novel coding SNP T560M in the Zurich case-control study (see discussion)

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No association of two functional polymorphisms in human ALOX15 with myocardial infarction

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ABSTRACT

The 12/15-lipoxygenase plays a janus-role in inflammation with pro-inflammatory and anti-inflammatory effects in cell systems and even opposite effects on atherosclerosis in two different animal species. Screening of the human 15-lipoxygenase (ALOX15) gene detected a polymorphic C to T substitution at position c.−292, which led to three times higher ALOX15 activity in macrophages and showed a trend to be atheroprotective in a small case–control study for coronary artery disease (CAD). A second polymorphism at position c.1693C > T leading to an T560M exchange and an inactive enzyme was recently associated with increased CAD. We now investigated whether these polymorphisms or a certain haplotype of ALOX15 are associated with myocardial infarction (MI) in a case–control subset from the population-based MONIKA/KORA cohort S3. Six polymorphisms in ALOX15 were analyzed in 2629 participants to cover all major haplotypes with a frequency higher than 1% in the Caucasian population. None of the polymorphism was associated with MI but a rare ALOX15 haplotype showed a significant protective effect on the risk for MI ($p = 0.03$). However, none of the polymorphisms or haplotypes was associated with CRP levels. These data suggest that ALOX15 may play a less prominent role during later stages of atherosclerosis involving atherothrombotic mechanisms than eventually during early plaque development.

1. Introduction

A wealth of information emerged about the involvement of the 12/15-lipoxygenases in atherosclerosis with several of its mechanisms ascribed to pro-atherosclerotic pathways (for review see Ref. [1]). The reticulocyte-type 12/15-lipoxygenase directly oxygenates linoleic acid esters on LDL particles leading to the formation of oxLDL and to foam cell formation [2–6]. On the other hand, there is compelling evidence for an anti-inflammatory and anti-atherosclerotic effect of the 12/15-lipoxygenases through the generation of lipid mediators involved in the resolution of inflammation [1]. The 12/15-lipoxygenase is involved in the metabolism of at least four classes of such pro-resolution lipid mediators, including the production of 15-HETE and the lipoxins from arachidonic acid [7], of 13-HODE from linoleic acid [8,9] and of the resolvins from DHA [10].

Animal models of atherosclerosis did not solve the question of whether the 12/15-lipoxygenase activity is pro- or anti-atherogenic because two different animal models showed contrasting results [11–15]. The discrepancies observed between different animal

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models have been explained by the different positional selectivities of the mammalian 12- and 15-lipoxygenase iso-enzymes which oxidize arachidonic acid at the carbon atoms 12 and 15 and which have different expression patterns [1].

In light of these contradicting reports and the equivocal results in two animal models of atherosclerosis [11–15], it will be essential to dissect the effect of the human reticulocyte 15-lipoxygenase (ALOX15) on human atherosclerosis for cardiovascular risk assessment in humans. Since functionally relevant polymorphisms in the ALOX15 gene could elucidate whether altered ALOX15 activity does influence disease progression in humans, we recently screened the ALOX15 gene for polymorphisms and detected 11 variations of which five defined all major haplotypes with frequencies >0.01 [16]. One of the polymorphisms, a C to T substitution at position c.−292 in the ALOX15 promoter, created a novel transcription factor binding site for the myeloid specific transcription factor SPI1 [16]. Binding of SPI1 to the c.−292T promoter increased ALOX15 transcription and activity in macrophages from heterozygous c.−292C > T carriers [16,17]. This functional polymorphism showed a trend to be atheroprotective in a small case–control study for coronary artery disease (CAD) [17]. In addition, a recent study showed evidence for another rare polymorphism in ALOX15 to be associated with CAD in two independent samples [18]. This T560M ALOX15 polymorphism with a minor allele frequency of 1.2% in Caucasians was shown to have 20 times reduced enzyme activity and was associated with an increased risk for CAD. Hence, both studies investigating the association with either an activating or an inactivating polymorphism in human ALOX15 suggest that the physiological ALOX15 expression does not accelerate atherosclerosis but protects from atherosclerosis.

In this study, we investigated whether any of the functional polymorphisms or a haplotype of the ALOX15 gene is associated with myocardial infarction (MI) in the MONIKA/KORA cohort S3 and in the KORA B study, a large case–control study including 518 cases and 2111 controls.

2. Materials and methods

2.1. Study population

Cases were derived from the KORA B study on patients with myocardial infarction (MI). In 1996, a total of 589 patients with history of sporadic MI prior to the age of 60 years were identified through the Augsburg, Germany, MONIKA MI register and invited to the study centre. The diagnosis of MI was established according to the MONIKA diagnostic criteria. MI patients were studied by physical examination, blood testing, echocardiography, and a detailed standardized interview. Controls were defined as study participants without MI from the population-based KORA study S3, which has been conducted during the years 1994/1995 in the region of Augsburg. Study participants were selected based on local registries and invited to undergo medical examination in the KORA study centre [19].

2.2. Genetic analysis

Genotyping was performed as previously described [20,21]. Briefly, genomic DNA was extracted from whole blood by standard salting-out method. High quality DNA for genotyping was obtained from 518 MI cases and 2111 controls. Genotyping of SNPs c.−635A > G (rs2255888), c.−186G > C (rs2664593), c.337 + 8C > T (rs11078528), c.1693C > T (rs34210653, T560M) and c.2076 + 200C > T (rs11568131) in ALOX15 was done at the Genotyping Unit of the Gene Discovery Core Facility at the Innsbruck Medical University, Austria, using a 5′ nuclease allelic discrimination (Taqman) assay in all subjects with sufficient amount and quality of DNA [21]. The MassArray system (Sequenom, San Diego, CA) was used for genotyping of c.−292C > T (rs11568070) whereby a base-specific extension reaction was performed with Thermosequenase (Amersham, Piscataway, NJ) and this reaction was dispensed onto a 384-format Spectro-Chip (Sequenom) [20]. A matrix-assisted laser desorption ionization time-of-flight mass (MALDI-TOF) spectrometer, model Bruker Autoflex (Sequenom), was used for data acquisition. Genotyping calls were made in real time with MassArray RT software (Sequenom). Calling rates were above 95% for all SNPs and did not differ between cases and controls for an individual assay.

2.3. Statistical analysis

Differences in study characteristics were tested either through non-parametric Mann–Whitney *U* tests for continuous variables and χ^2 tests or Fisher's exact test for categorical variables. SNP association analysis was performed under the assumption of an additive genetic effect model. Association with MI was analyzed through logistic regression models, association with CRP plasma levels through linear regression (on the log scale for CRP). All association analyses were adjusted for age and sex. Haplotype estimation was performed using "haplo.em" from R library "haplo.stats" [22] stratified by case–control status and only for individuals with complete genotype information in all SNPs. A likelihood ratio test to test for differences in haplotype frequency estimates between cases and controls was applied. For haplotype analysis, the expected number of copies of all haplotypes with frequency ≥1% were calculated and coded as covariates. Haplotypes with probabilities <1% were collected into a group of rare haplotypes. All haplotype variables (including the group of rare haplotypes) were modelled against the group of subjects with two copies of the most frequent haplotypes (reference). All haplotype analyses were adjusted for sex and age alone or additionally for waist, HDL-C and hypertension. Significance was defined for *p*-values below 0.05.

3. Results

To investigate the influence of ALOX15 on atherosclerosis, we analyzed the association of six polymorphisms including the functional polymorphisms c.−292C > T and T560M in ALOX15 with MI in the MONIKA/KORA case–control sub-study including 518 Caucasian cases and 2111 healthy controls. The characteristics of the controls and cases are shown in Table 1. No significant association between the detected genetic variants in the ALOX15 gene and MI was observed (Table 2) but the rare functional polymorphism T560M leading to reduced ALOX15 enzymatic activity showed a trend to increase the risk of MI (OR 1.7, CI: 0.96–3.01, $p = 0.06$). A similar trend for an association was observed when the analysis of the T560M polymorphism was restricted to men only (OR = 1.9, CI: 1.0–3.7, $p = 0.05$).

Haplotype analysis of the ALOX15 gene in the MONIKA/KORA cohort revealed nine previously defined haplotypes (Table 3) with similar frequencies to the ones found in a Swiss study population [16]. Since the T560M polymorphism was only observed in rare haplotypes with a frequency below 1% in the entire population and no further contribution to haplotype association analysis was expected from this SNP, we excluded this SNP from haplotype estimation, in order to increase the number of individuals with comprehensive genotype information. Logistic regression analysis adjusted for age and sex showed a significant association of haplotypes 4 and 8 with MI ($p_{haplo.4} = 0.02$, $p_{haplo.8} = 0.03$). After the additional adjustment for waist, hypertension, and HDL-C only haplotype 4 remained significantly associated with MI while the association for haplotype 8 became borderline significant ($p_{haplo.4} = 0.03$, $p_{haplo.8} = 0.05$,

Table 1
Characteristics of controls and cases.

	Controls (n = 2111)	Cases (n = 518)	p-Value
Age (years)	56.0 (51.0; 62.0)[a]	58.0 (54.0; 61.0)	0.0002
Male (%)	49.3	87.6	$<10^{-20}$
Hx of diabetes (%)[b]	6.3	16.5	$<10^{-13}$
Hx of hypertension (%)[b]	50.7	64.5	1.8×10^{-8}
Lipid lowering medication	4.4	41.7	$<10^{-20}$
Smoking			2.2×10^{-16}
Non-smokers	50.9	12.5	
Ex-smokers	29.4	65.1	
Current smokers	19.8	22.0	
Waist (cm)	93.0 (84.0; 100.9)	101.0 (94.5; 108.0)	$<10^{-20}$
Hip (cm)	104.0 (99.5; 109.0)	102.0 (98.0; 107.0)	7.3×10^{-7}
Alcohol (g/day)	5.7 (0; 24.6)	8.7 (0; 25.7)	0.1720
Cholesterol (mg/dl)	238.6 (212.1; 266.7)	235.6 (206.8; 268.7)	0.1541
HDL-C (mg/dl)	51.0 (42.0; 62.4)	48.0 (40.5; 58.6)	4.0×10^{-5}
LDL-C (mg/dl)	148.0 (122.9; 176.0)	139.7 (112.7; 168.4)	8.3×10^{-6}
Total C/HDL-C ratio	4.7 (3.7; 5.8)	4.9 (4.0; 5.8)	0.0120
CRP (mg/l)	1.6 (0.8; 3.2)	2.2 (1.1; 3.9)	9.4×10^{-9}

[a] Median (interquartile range).
[b] Hx of [. . .] = history of [. . .].

Table 2
Exact logistic regression models predicting case-status (MI).[a]

Variation	MAF (%) genotypes		Odds-ratio[a]	95% CI	p-Value
	Controls (n = 2111)	Cases (n = 518)			
c.−635A > G rs2255888	25.3 1176/794/135	23.4 308/174/34	0.96	0.81–1.14	0.63
c.−292C > T rs11568070	1.7 2038/69/1	1.7 501/16/1	1.04	0.58–1.76	0.89
c.−186G > C rs2664593	22.1 1240/700/101	21.4 310/155/28	0.98	0.82–1.18	0.84
c.337 + 8C > T rs11078528	27.3 1112/841/154	29.0 264/205/47	1.07	0.91–1.26	0.41
c.1693C > T rs34210653	1.3 1976/51/1	2.0 458/19/0	1.73	0.43–2.30	0.06
c.2076 + 200C > T rs11568131	16.5 1417/546/60	14.5 359/116/13	0.88	0.71–1.08	0.21

[a] Age and sex adjusted using an additive genetic effect model.

data not shown). However, no overall significant difference between haplotype frequencies in cases and controls was found (p = 0.23).

To explore whether these haplotypes modulate the anti-inflammatory role of ALOX15, we performed linear regression analyses to investigate the influence of the ALOX15 haplotypes on CRP levels. No haplotype and no individual polymorphism were associated with CRP levels without or with adjustment for case status, age, sex, waist, hypertension, lipid lowering drugs, and HDL-C (data not shown).

Table 3
Predicted haplotypes and their frequencies in the study[a] and results from logistic regression models predicting case-status (MI).[b]

Haplotype	Alleles[a]	Frequency in		OR	95% CI	p-Value
		Controls (n = 1997)	Cases (n = 486)			
14	ACG**T**C	0.254	0.278	–	–	–
10	ACGCC	0.243	0.254	1.002	0.811–1.237	0.98
8	AC**C**CC	0.176	0.175	1.305	1.027–1.656	0.03
5	**G**CG**T**	0.119	0.112	1.205	0.920–1.571	0.17
11	**G**CGCC	0.111	0.094	1.080	0.805–1.442	0.60
4	ACG**T**	0.022	0.012	0.408	0.180–0.826	0.02
1	AC**CT**	0.019	0.013	0.705	0.328–1.389	0.34
12	A**T**GCC	0.016	0.016	1.078	0.581–1.903	0.80
9	**GC**CC	0.016	0.009	0	0–0	0.96
Rare[c]		0.013	0.016	5.999	2.884–12.635	<0.001

[a] Haplotypes formed by the alleles of the ALOX15 polymorphisms c.−635A > G, c.−292C > T, c.−186G > C, c.337 + 8C > T, c.2076 + 200C > T. Rare alleles are printed in bold.
[b] Adjusted for age and sex.
[c] Haplotypes with frequencies < 0.01 were pooled in a group as rare haplotypes.

4. Discussion

ALOX15 was previously shown to have contrary effects on atherosclerosis in different animal models, which complicated the interpretation of the role of the enzyme in human atherosclerosis. We and others previously identified two rare functional polymorphisms in ALOX15, which led to increased (c.−292C > T) [16] and reduced (T560M) [18] enzyme activity, respectively. While the activating c.−292C > T polymorphism showed a trend towards an atheroprotective effect in a case–control study for CAD, the inactivating T560M polymorphism was associated with a significantly increased risk for CAD, indicating that ALOX15 is anti-inflammatory and anti-atherogenic in humans [17,18]. In the present study, we investigated for the first time the role of ALOX15 on the emergence of MI and show that the functional polymorphisms are not associated with MI in a larger Caucasian case–control sample, suggesting that ALOX15 does not play a major role during later stages of atherosclerosis involving atherothrombotic events.

Such a divergent role of ALOX15 during different stages of atherogenesis would be supported by the temporal and regiospecific expression of ALOX15 and by the expression of its specific linoleic acid metabolite in the arterial wall. In rabbit atherosclerotic lesions, the expression of ALOX15 was 10 times higher in early lesions than in more advanced lesions [23] and the specific ALOX15 metabolite 13(S)-hydro(per)oxy-octadecadienoic acid (13-HpODE) was abundant in early human atherosclerotic lesions but was not during later stages of plaque development [8,9]. In line with this, a recent human genetic study suggested a role for ALOX15 only during early atherosclerotic lesion development. This study investigated the association of three ALOX15 promoter polymorphisms with carotid plaques and found an association of a certain promoter haplotype only in the subgroup of the younger stratum, while this haplotype was not associated with the intermediate phenotype in the older subgroup [24]. Therefore, it is conceivable that in humans ALOX15 may play an anti-inflammatory role in the initiation of atherosclerosis but to a lesser extend during later stages of atherogenesis, although ALOX15 expression during human atherosclerosis is under debate [1].

There is another possible interpretation of this negative finding. There could be an effect of these rare polymorphisms with an OR of 1.5 or less, which our study could not detect because it was underpowered (39%). We had a power of 87% to detect an effect of OR = 2.0 for a SNP with MAF comparable to c.−292C > T and T560M (MAF = 1.4%). An indication for a lack of power of the study could be the observed trend for an association of the T560M polymorphism with MI with a similar risk increase (OR 1.7) as previously reported for CAD in the CARDIA and the ADVANCE studies [18]. This raises the question why the second functional promoter polymorphism, c.−292C > T, would not show a similar trend for an association with MI. This discrepancy may derive from the tissue specific influence of the two functional polymorphisms on ALOX15 activity. While the T560M polymorphism will result in reduced ALOX15 activity in all cell types [18], the c.−292C > T promoter polymorphism only leads to increased ALOX15 expression in myeloid cells [16]. There is indication that endothelial expression of ALOX15 will result in the formation of arachidonic acid metabolites leading to vasodilation [25–27], which would only be influenced by the T560M polymorphism and not by the c.−292C > T promoter polymorphism. Therefore, the trend of the T560M polymorphism for an association with MI might indicate that endothelial expression of ALOX15 could be involved during the later atherosclerotic events leading to MI. On the other side both of these findings could derive from chance.

To get additional information on whether this M560 ALOX15 allele is associated with CAD, we investigated its association with angiographically documented CAD in a smaller case–control study involving 498 participants [17,28,29]. Again, the T560M polymorphism showed a trend to be associated with increased risk for angiographically defined CAD in this small study (OR 3.30 [CI: 0.68–16.0]; $p = 0.14$) potentially indicating some role as a risk marker for CAD (data not shown).

The inactivating M560 allele was not linked with a specific haplotype but was found on four different haplotypes including the three frequent haplotypes 8, 10 and 14 while being absent on haplotype 4. These results show that none of the functional polymorphisms in ALOX15 do account for the association of haplotype 4 with MI in the MONIKA/KORA case–control study, leaving the possibility of the presence of another atheroprotective mutation linked with this haplotype or of a chance finding.

In summary, we show that the two functional polymorphisms in ALOX15 are not associated with MI in a large case–control study which argues that ALOX15 seems less involved during the later stages of atherosclerosis than eventually during early lesion development. Indeed, these activating and inactivating polymorphisms in the ALOX15 gene seem inversely associated with CAD and suggest an anti-inflammatory role for ALOX15 during early lesion development.

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3 DISCUSSION

Human ALXR is expressed in almost all leukocytes [90, 93, 94, 224] and is involved in inflammatory disease such as rheumatoid arthritis and asthma [165] where it influences cell infiltration into tissue. ALXR function is mediated by a variety of pro- and anti-inflammatory ligands; all of them are important stimuli of inflammation. However few is known about its own transcriptional and translational regulation in monocytes and in macrophages. In this thesis we aim at better understand the transcriptional regulation of the ALXR in macrophages and its exact role during inflammation.

In this study ALXR expression has been shown to be tightly regulated during monocytes differentiation into macrophages. ALXR expression in monocytes is confirmed by previous studies in which the ALXR ligand LXA_4 was shown to recruit monocytes to the site of inflammation without increasing their cytotoxicity and superoxide generations [32, 93]. These monocytes are indeed differentiated into pro-resolving M2 macrophages, which take up apoptotic cells from the inflamed tissue. Therefore high expression of ALXR in these cells reflects its possible role as an anti-inflammatory chemotactic receptor for cells involved in the late phase of inflammation. However ALXR expression is almost absent in macrophages. Only increased levels of IFNγ could rescue the loss of ALXR transcription during monocytes differentiation into macrophages. Hence macrophages might need to be in a stimulatory environment of IFNγ to activate transcription factors responsible for ALXR expression.

IFNγ is an endogenous mediator of immunity and inflammation and plays a complex and central role in the resistance of the mammalian host to pathogens. Although originally defined as an agent with direct antiviral activity [225], the properties of IFNγ include stimulation of bactericidal activity of phagocytes [226], stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules[227], orchestration of leukocyte-endothelium interaction [228], effects on cell proliferation and apoptosis [229], activation of macrophages and T helper 1 (Th1) cell responses as well as stimulation and repression of a variety of genes [230]. IFNγ is a dimerized soluble protein that is secreted by T-lymphocytes and NK cells [230]. Indeed, the pro-inflammatory Th1 cells secrete IFNγ [231], which induce the development of classical activated M1 macrophages. M1 macrophages display enhanced endocytic functions and have the ability to kill intracellular pathogens [232, 233]. Such M1 type-macrophage showed specific M1 markers like the T-cells costimulatory proteins CD86 and CD 80 [234]. Since IFNγ has already been shown to induce the expression of the mouse homologue of ALXR in microglial cells [235], it may induce in

M1 macrophages the expression of ALXR to mediate pro-inflammatory actions. In fact, in epithelial cells IFNγ increased the expression of the ALXR specific ligand SAA [236], which is increased by as much as 1000 fold in response to acute infection [99] and has been shown to provoke a marked increase in the number of PMNs in a mouse air-pouch model [158]. These authors have also shown that this effect was abrogated in $ALXR^{-/-}$ mice confirming that SAA binds to the ALXR to enhance the recruitment of PMNs into inflamed tissue [158]. In addition SAA has been shown to stimulate monocytes via ALXR to produce the pro-inflammatory chemokines CCL2 [132] and TNFα [237]. Furthermore, in neuroblastoma cell lines, IFNγ in combination with TNFα upregulates Aβ42 formation [238], which has a central role in neurotoxicity and has been shown to induce chemotaxis of mononuclear phagocytes via ALXR in brain tissue of Alzheimer patients [239]. In consequence, IFNγ effect on the expression of ALXR might initiate ALXR to have pro-inflammatory properties following SAA triggering, such as generation of inflammatory chemokines and cytokines and the induction of chemotaxis of pro-inflammatory cells.

In contrast, treatment with ANXA1 and Ac2-26 of acute inflammation in a mouse model inhibited recruitment of PMN, which was markedly attenuated in $ALXR^{-/-}$ mice [158]. Thus, ALXR also signals anti-inflammatory effects and inhibits migration of PMNs when pro-resolving mediators are present. Indeed, Ac2-26 was also shown to inhibit SAA-induced chemotaxis of macrophages [158] and another anti-inflammatory ligand, LXA_4, was shown to promote chemotaxis of monocytes without leading to cytotoxicity but to nonphlogistic phagocytosis of apoptotic PMNs [32, 33]. Hence, upregulation of ALXR in macrophages by IFNγ might also lead to resolution of inflammation and tissue repair, when the anti-inflammatory ligands ANXA1, Ac2-26 or LXA4 rise in concentration during the resolution phase of inflammation.

These data support that during inflammation, the ALXR plays a role in chemotaxis of monocytes, which results from a gradient of pro-inflammatory or anti-inflammatory ligands. Depending on the ligand and the environment of the invaded tissue, the monocyte may then differentiate into either an M1 or a M2 macrophage. Since IFNγ is known to induce the differentiation of macrophages into pro-inflammatory M1 type macrophages, these results suggest that in the early phase of inflammation only M1 macrophages could further be triggered by the pro-inflammatory ligands of ALXR to generate inflammatory regulators and to recruit PMN and monocytes that recognize and kill pathogen. On the other hand, when anti-inflammatory ligands occur at the site of inflammation, these M1 macrophages may re-differentiate into M2 macrophages, and play a role in restoring tissue homeostasis. Hence, in monocytes ALXR is probably mediating chemotaxis while in a limited set of M1 macrophages first pro- and then eventually anti-inflammatory effects may be triggered depending on the local environment.

Up to date there are no studies that have characterized the ALXR promoter. In this thesis we have defined different 5'UTR of ALXR mRNA in macrophages and found two distinct transcriptional start sites separated from each others by 224bp. These mRNAs contain both the coding exon (third exon) with one having a longer version of first exon. 5'UTRs play a role in translational regulation and conservation of important UTRs between species has been shown before [240]. In this line it is notable that part of the long version of the first ALXR exon is conserved between mouse, dog, and human, suggesting a possible regulatory role of this region. Furthermore it has been estimated that 10-18% of genes express alternative 5'UTRs by using multiple promoters [241, 242]. Multiple transcriptional start sites is a result of either a variation in the initiation site from one promoter or of the use of separate promoters [243]. To test which promoter configuration is present in the ALXR gene, fragments of putative promoter constructs were designed upstream of the two transcriptional start sites and tested for their transcriptional activity. Constructs were built so that potential downstream elements were included not to miss important elements like DNE [244]. From the results obtained, we can conclude that ALXR expression is driven by two distinct promoters, P1 and P2. Both promoters lack a TATA boxes at position -25 but MatInspector and Alibaba algorithm identified putative basal transcription binding sites in promoter P1 and P2.

P1 contains an OCT1 binding site which has been previously shown to play a critical role in preinitiation complex recruitment of promoters that lack TATA boxes. For instance during the transcription of the lipoprotein lipase, OCT1 was able to replace the TBP via its interaction with the general transcription factor TFIIB [245]. Therefore, such an OCT1-TFIIB preinitiation transcription complex may help to recruit the RNA polymerase II and to drive transcription of the ALXR via its first promoter P1. Or, like in other cases, OCT1 could act in synergy with recruited transcription factors of the same family [246] to upregulate ALXR transcription. Similarly P2 contains the putative basal transcription factor binding site for SP1, which is known to bind CpG islands and to drive gene transcription of TATA less promoters [247]. Indeed SP1 has two glutamine rich transactivator domains A and B, which each interact with the TATA-binding protein TBP and with TFIID [248] to recruit the basal transcriptional machinery of RNA polymerase II [249-251]. Furthermore, SP1 can either be transactivated via the SP1 binding sites [252] or can be superactivated with other DNA-bound SP1 molecules. Further investigation on other putative transcription factors upstream of the first promoter and/or on transcription factor protein complexes would give us a greater view on the transcriptional machinery recruited to the ALXR promoters and its regulation. The existence of two distinct promoters is a sign of different use of gene regulation according to environment and stimuli.

IFNγ binds to the heterodimeric receptor consisting of the Interferon γ receptor 1 and 2 (IFNGR1 and IFNGR2) [253] and either activates the JAK/STAT pathway in which STAT proteins translocate into the nucleus or induces transcription of Interferon Regulatory Factors (IRF) [254]. Both of these pathways enhance gene transcription in response to IFNγ. The first promoter of ALXR seems to respond to IFNγ and contains several interferon response elements. Since the different constructs of the first promoter are equally stimulated by IFNγ, the shortest version of first promoter was investigated for its putative interferon response element. MatInspector revealed that IRF4 and IRF7 binding sites were present in the ALXR promoter. Even though mutation in the binding site of IRF4 reduced DNA-protein binding and promoter activity, IRF1 was responsible for the IFNγ response and not IRF4. All IRFs bind to a consensus sequence $^{A}/_{G}$NGAAANNGAAACT corresponding to the IFN-stimulated response element IRSE [255]. IRF1 is one of the best characterized and the highest induced member of the IRF family. For instance, it was shown that THP-1 monocytes stimulated with IFNγ resulted in a strong increase in IRF1 binding to the IRSE element in the β2-microglobulin promoter [256]. In this study we show that the first ALXR promoter P1 contains an IRSE element which is bound by IRF1 when THP-1 macrophages are stimulated with IFNγ. The EMSA experiments also indicated that IRF1 is complexed with other proteins. Indeed IRF members do not work alone but synergize with other proteins to drive regulated transcription of genes [257, 258]. Taken together, these data indicate that the ALXR promoter contains an IRSE transcription factor binding site, which is bound by IRF1 to induce transcription upon IFNγ stimulation.

However in this study we had some unsolved results concerning the expression of the ALXR in macrophages following IFNγ stimulation. Firstly, IFNγ stimulation resulted in a 80-fold increase of ALXR mRNA expression but only in a 1.4-fold increase of the luciferase constructs with promoter P1. Such different results indicate that the IRSE binding site in the first promoter may be not the only transcription factor binding sites that respond to IFNγ. Other genes like IFNβ have enhancer elements far upstream of their promoter that increase transcription of their mRNA [259]. Accordingly, the ALXR promoter may contain far upstream enhancers that respond to IFNγ and that increase ALXR gene transcription eventually in concert with IRF1 binding site.

Secondly, we were able to detect the ALXR protein in monocytes by FACS analysis and could confirm its absence in macrophages. However, we could not detect the ALXR protein by FACS in macrophages stimulated with IFNγ. This may be due to technical problems of detaching macrophages from cell culture dishes, although we could detect the presence of other cell surface receptors on macrophages (data not shown). Western blot analysis could also not solve the question

of expression of ALXR on IFNγ stimulated macrophages because no specific antibodies are available, although we tried several commercial ones. Corroborating the lack of a specific antibody is the observation that no study published a western blot of the ALXR protein so far.
On the other hand, the lack of ALXR protein in macrophages stimulated with IFNγ may indicate that post-transcriptional mechanisms regulate ALXR abundance on the cell surface. INFγ stimulated transcription of the ALXR leads presumably to the mRNA containing the longer 5'UTR because only the first promoter contains the IRSE element and is responsive to IFNγ stimulation. Indeed 5'UTRs were shown to contain regulatory motifs [260, 261] that have an effect on translational efficiency. Among the potential regulatory mechanism that could modulate translation efficiency are the presence of target sequences for MicroRNAs [262], internal ribosome entry sites [263], hairpin secondary structures [264], upstream translation initiation codons (uAUG) and upstream open reading frames (uORF). Intriguingly, the longer 5'UTR of the ALXR mRNA derived from promoter P1 contains an uORF which may lead to a poor translation of this ALXR mRNAs [265]. A recent study revealed that the presence of an uORFs in human genes results in a 30-80% reduction in protein expression [265-267] or even in a full block of translation. Hence, IFNγ stimulation of macrophages may result in ALXR mRNA containing the uORF, which may not be efficiently translated and therefore not support membrane expression of the receptor.

Polymorphism in the ALXR gene could alter transcription or lead to amino acid changes which could influence progression of inflammation. To investigate whether the ALXR plays a role in chronic inflammation, we searched the gene for polymorphisms and investigated the association of the polymorphisms with coronary artery disease (CAD), as a model for an inflammatory disease (see appendix, supplementary methods). We screened the promoter, the coding region and the 3'UTR of ALXR from 98 Caucasians for polymorphisms by DHPLC and found 8 mutations. Five of these polymorphisms were located in the promoter region, one in the coding region and two in the 3'UTR (see appendix, supplementary Table 3). Since 3 mutations (c.-7978 A>G, c.-7986 A>G; c.-7893C>T) are located in the first promoter region P1 and mutations in promoters could influence transcriptional activity of a gene [268], we tested their effect in reporter assays (see appendix, supplementary Figure 1). However none of them altered the transcriptional activity of the first promoter P1 in macrophages. Furthermore, no SNPs were observed in the second promoter of the ALXR. To investigate whether the ALXR is associated with CAD, we analyzed the association of these SNPs in a case-control study involving 238 healthy volunteers and 260 patients with angiographically documented CAD [213, 215, 269]. No significant association was observed for any of the polymorphisms investigated, although the SNP c.-7978A>G showed a trend for a

proatherogenic effect (OR= 1.4; 95%CI; p=0.27) (see appendix, supplementary Table 3). From the results obtained, we cannot exclude that there are functional polymorphisms upstream and downstream of the regions that were screened. However the absence of functional polymorphisms in the regulatory and coding sequences of ALXR suggest that this gene may be important for the correct function of cells during chronic inflammation.

In this study, we have described for the fist time the regulatory sequences and the potential transcriptional machinery that enhance the expression of the ALXR gene in macrophages. Furthermore our data support that in monocytes, ALXR is probably mediating chemotaxis while in a limited set of M1 macrophages first pro- and then eventually anti-inflammatory effects may be triggered depending on the local environment. Hence this study provides a better insight of the role of the ALXR in the inflammatory process and stimulates research in the function of ALXR during chronic inflammatory disease

4 APPENDIX

4.1 **Supplementary methods**

Case-control study

339 men and 159 women in Zürich volunteered to participate in the study. Written informed consent was obtained from all participants and the local ethics committees approved the study. The sample consisted of 260 consecutive Caucasian patients with angiographically documented coronary artery disease with more than 50% stenosis in at least one coronary artery. The control group consisted of 238 Caucasians with no history of CAD, stroke, or peripheral vascular disease and was recruited from the general population. Angiographically negative individuals were also included in the control group. 81% of the cases and 8% of controls were under cholesterol lowering regimen [213, 269].

Denaturing High-Performance Liquid Chromatography (DHPLC) Analysis

DNA was extracted from EDTA-supplemented blood samples with the QIamp® DNA Blood Mini Kit (Qiagen AG, Hombrechtikon, Switzerland). PCR was carried out in 50µl reaction volumes containing 500ng of DNA. The primers (Microsynth, Balgach, Switzerland) used for amplification, the size of the amplicons and the respective annealing temperature are listed in the supplementary data (supplementary Table 1). The PCR products were denaturated for 2min at 94°C and cooled to 60°C for reannealing at a temperature ramp rate of 1°C/min. DHPLC was carried out on a WAVE DNA fragment analysis system (Transgenomic) [270]. All samples that showed a unique absorption pattern were sequenced.

Tetra-primers Analysis

For each amplification a total volume of 25µl with 150ng of blood DNA, 0.2mM of deoxynucleotide triphosphate mixture (dNTPs), 1.5mM of $MgCL_2$ (Solis BioDyne,), 1U of HOT FIREPol DNA polymerase (Solis BioDyne,) and titrated amounts of 10 µM of forward and reverse primer were used (supplementary Table 2) [271]. PCR reactions were performed under the following conditions: a denaturation step for 10min at 95°C followed by the first set of cycles: denaturation for 30sec at 95°C, annealing for 30sec at the appropriated annealing temperature and extension for 7sec at 72°C. In the allele-specific amplication, DNA was denaturated for 30sec at 95°C, annealed for 30sec at the appropriated temperature and extended for 7sec at 72°C. A final extension for 7 min at 72°C was added and PCR were visualized on a 2% agarose gel.

Statistical analysis

Association with CAD was analyzed through logistic regression models, using an additive genetic model and adjusted for age and sex

4.2 **Supplementary data**

4.2.1 Supplementary Table 1

PCR Nr.	Primer sequence (5 '-3')	Boundaries forward *reverse*	Amplicon Size (bp)	PCR Annealing Temp. °C	DHPLC Oven Temp. °C	% Buffer B Start-End
1	GGGAACATCACACTCTGGGGAC CGAAAGGGGTCACAAAAAAGCA	g.56954509 *g.56954871*	362	57	59/60/64	55-70
2	GAAATTGCCTCTGCCGCCCTCT AGACAGATGCCTGGCTCCTT	g.56954767 *g.56955037*	270	55	60/61/62	55-70
3	GCCTACATGTTCCCTCCTCC CAACAGAGCGAGACTCCATC	g.56954941 *g.56955307*	366	55	62	55-70
4	ATGTAGGTAATGTGCCTTCT CCTTCCTCTGTTCTGATAAT	g.56955239 *g.56955630*	391	55	63/64/65	55-70
5	AGCGCCCAGCCAGTAATGTG GTTTCAGCCACGCAATCAAG	g.56955560 *g.56955947*	387	55	60/64	55-70
6	CACATTTGATGCCAGAAGTA CCTGTGTGCTTCTTGGCTTCCTGT	g.56955863 *g.56956276*	413	55	59/64	55-70
7	GTAGGAGTGGGAGCTTTAGTGGA TAATGTGGCCGTGAAAGAAAAGT	g.56963610 *g.56963957*	347	58	56/60/64	55-70
8	TGCTTGGGGTCACCTTTGTC CCACCTTCAGCCTCTCCTCA	g.56963818 *g.56964303*	485	58	61	55-70
9	GCCATGAAGGTGATCGTCGG GACGTAAAGCATGGGGTTGAGG	g.56964147 *g.56964632*	485	58	56/60/64	55-70
10	CTGGGCACCGTCTGGCTCAA CAAGGGTAGATGAAGCTGGAACTGG	g.56964510 *g.56964850*	340	58	57/61/65	55-70
11	CACGGCTGCCAATTCTGCTTC TTTAAAGTCTTCCCCTTATTATGCGCTATC	g.56964725 *g.56965093*	368	58	54/58	55-70
12	GTTTTTTGACTTCTGCCTATACCCTG AAATTCTAACCAACTCCTGTGAGATATAAAT	g.56964974 *g.56965409*	435	53	51/55/59	55-70
13	ATGCATTTTCTACATGTATTAAGATGGTCA CGCACCCAGCCAAGTTTCAT	g.56965234 *g.56965826*	592	52	50/54/58	55-70

Table 1: Primer sequences for ALXR amplification, PCR conditions and parameters for DHPLC analysis

4.2.2 Supplementary Table 2

Position -SNP	Primer sequence (5 '-3')	µ l in 25µl total	(°C)	(°C)	Amplicon size (bp)
c.-8751 A>G					
Forward outer primer	TGCTTATTGCTGTCTGCCTT	1.5			Two outer primer: 671 bp
Reverse outer primer	ATGAAACCCCACCTCTACTA	1.5	64	47	
Forward inner primer (A allele)	CGGATATTGACTCTA	2	(18x)	(20x)	A allele: 510 bp
Reverse inner primer (G allele)	GATTCACGGATCC	0.5			G allele: 188 bp
c.-8461 T>G					
Forward outer primer	GCCTACATGTTCCCTCCTCC	1			Two outer primer: 496 bp
Reverse outer primer	AGCTGGGCGTGGTG	1	64	45	
Forward inner primer (T allele)	GTAGGTAATGTGCCTTCTTCTTTT	1.5	(20x)	(25x)	T allele: 194 bp
Forward inner primer (G allele)	GTAGGTAATGTGCCTTCTTCTTTG	1.5			G allele: 194 bp
c.-7986 A>G					
Forward outer primer	TCATGATGTTGGCTAGGATGGTTTTC	1			Two outer primer: 486 bp
Reverse outer primer	AAGAGTTTCAGCCACGCAATCAAGCC	1	65- 69	45-55	
Forward inner primer (A allele)	ACAGTTTTGGGAGAGTGA	1	(20X)	(20X)	A allele: 230 bp
Forward inner primer (G allele)	ACAGTTTTGGGAGAGTGG	1			G allele. 230 bp
c.-7978 A>G					
Forward outer primer	TCATGATGTTGGCTAGGATGGTTTTC	1			Two outer primer: 486 bp
Reverse outer primer	AAGAGTTTCAGCCACGCAATCAAGCC	1	65- 69	45-55	
Reverse inner primer (T allele)	AGTGTCAGATCCCACAAGTT	1	(20X)	(20X)	A allele: 301 bp
Reverse inner primer (C allele)	AGTGTCAGATCCCACAAGTC	0.5			G allele. 301 bp
c.-7893 C>T					
Forward outer primer	CCACCCCCGATTTATAGCCA	0.5			Two outer primer: 580 bp
Reverse outer primer	CGCGGAGACTACTGAATGAT	0.5	59	44	
Forward inner primer (C allele)	TGTCCCCAGAGAAC	1.5	(16x)	(22x)	C allele: 408 bp
Reverse inner primer (A allele)	AAGCAATTCTCCAA	1.5			T allele. 199 bp
c.410 G>A					
Forward outer primer	CATTACCATTCCTCATTGTCT	0.5			Two outer primer: 536bp
Reverse outer primer	AAACCAACA-GATGAAGAAAGA	0.5	56	36	
Forward inner primer	GCCCAGAACCACCA	1	(15x)	(27x)	G allele: 196bp
Reverse inner primer	GCCAGACTCACAGTGC	0.2			A allele: 369bp
c.1315 A>G					
Forward outer primer	CTGCCAATTCTGCTTCACCTCCTG	1			Two outer primer: 450 bp
Reverse outer primer	AGCAAACTAAAAATAGAAGAAAACTT	1	60	50	
Forward inner primer (G allele)	AGAAGAGAAAGACCG	0.5	(15x)	(20x)	G allele: 156 bp
Reverse inner primer (T allele)	TTACAAATCCCCACT	2.5			A allele. 323 bp
c.1346 C>T					
Forward outer primer	CTGCCAATTCTGCTTCACCTCCTG	1			Two outer primer: 450 bp
Reverse outer primer	AGCAAACTAAAAATAGAAGAAAACTT	1	60	50	
Forward inner primer (T allele)	TAAGACTTAGATGAGATAGT	2	(15x)	(20x)	T allele: 130 bp
Reverse inner primer (G allele)	TCCCCTTATTATGCG	2			C allele. 354 bp

Table 2: Primers for Tetra-primer to investigate ALXR promoter mutations

4.2.3 Supplementary Table 3

NM 01005738	Allele Frequency %		Control	Cases	Odds ratio	95%CI P value	P value
c.-8751 A>G rs11666254	A: 69.96	G: 30.04	47.90/44.12/7.98	47.50/41.30/11.2	0.993	0.687-1.437	0.972
c.-8461 T>G rs11670660	T: 68.65	G: 31.65	46.64/44.12/9.24	47.29/41.47/11.24	0.947	0.654-1.370	0.771
c.-7986 A>G rs35215887	A: 72.53	G: 27.47	50/45.8/4.2	51.74/40.93/7.33	0 .905	0.62-1.309	0.596
c.-7978 T>C rs35225016	A: 94.97	G: 5.03	92.86/7.14/0	88.42/10.42/1.16	1.434	0.7-2.751	0.2785
c.-7893 C>T rs56238033	C:89.26	T:10.71	77.08/22.92/0	77.7/20/2.3	1.094	0.702-1.7.03	0.692
c.410 G>A				Private mutation			
c.1315 A>G rs17695052	A: 89	G: 11	76.89/23.11/0	80.23/18.6/1.16	0.828	0.52-1.30	0.676
c.1346 C>T rs17695064	C:89	T:11	76.89/22.69/0.42	81.32/17.90/0.78	0.82	0.520-1.295	0.395

Table 3: Logistic regression models predicting CAD status for cases. Adjusted for age and sex.

4.2.4 Supplementary Figure 1

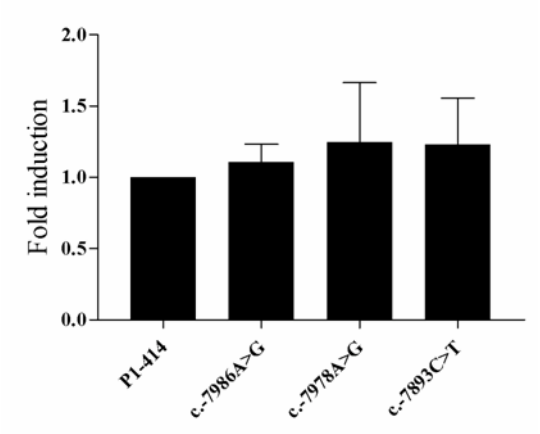

Figure 1: P1-414 basal transcription after SNPs site-directed mutagenesis. Luciferase expression of the P1-414 promoter with mutations introduced according to the promoter SNPs determined (c.-7986 A>G: A-to-G base exchange at position -7986; c.-7978 A>G: A-to-G base exchange at position -7978; c.-7893 C>T: C-to-T base exchange at position -7893). Human THP-1 monocytes were transfected with the constructs, differentiate into macrophages and luciferase activity was measured and normalized against the activity of the Renilla luciferase 48hrs later. All experiments were done in triplicate and bars indicate the means of three independent experiments with three plasmid preparations. Values represent fold induction compared to the P1-414 vector and the error bars represent the standard error (± SD). *p<0.05; **p<0.01

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SUPERVISING ACTIVITES

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LANGUAGES

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German	good knowledge - B1 of the European standards of languages

PUBLICATIONS

2009 **Atherosclerosis.2009 Jul; 205(1):192-6**
"No association of two functional polymorphisms in human ALOX15 with myocardial infarction"
Hersberger M, Müller M, Marti-Jaun J, Heid IM, Coassin S, Young TF,Waechter V, Hengstenberg C, Meisinger C, Peters A, König W, Holmer S, Schunkert H, Klopp N,Kronenberg F, Illig T

POSTER PRESENTATIONS

2006 **12th Cardiovascular Biology and Clinical Implications Meeting, Murten (Switzerland)**
Transcriptional control of the lipoxin receptor ALXR
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2007 **6th Day of Clinical Research, Zürich (Switzerland)**
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2007 **3rd ZHIP Symposium, Zürich (Switzerland)**
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2008 **Swiss Med Lab 2008, Montreux (Switzerland)**
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